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Induction of resistance to herpesvirus and calicivirus in the domestic cat by stimulation of the innate immune system

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Für Simon, Mami und Papi,
die mich alle immer unterstützt, gefördert und geliebt haben.

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Abbreviations

ABCD	European Advisory Board on Cat Diseases
ABL	v-abl Abelson murine leukaemia viral oncogene homologe
ACTB	β-actin
ADAR	RNA-specific adenosine deaminase
AE	amplification efficiency
Ag	antigen
AP1	transcription factor activator protein 1
APCs	Antigen-presenting cells
B2M	β-2-microglobulin
cDC	conventional dendritic cell
CDP	common DC precursor
CIS	cytokine-inducible SH2 protein
CIITA	MHC class II transactivator factor
CPE	cytopathic effect
CpG	cytosine phosphate guanine
CRFK	Crandell-Reese feline kidney cells
CTL	cytotoxic T cell
EDTA	ethylendiamintetraacetat
eIF-2α	protein synthesis initiation factor 2
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
ER	endoplasmic reticulum
FCoV	feline coronavirus
FCV	feline calicivirus
Fcwf-4	felis catus whole fetus cells
FCS	fetal calf serum
FeLV	feline leukemia virus
FHV	feline herpesvirus
FIV	feline immunodeficiency virus
DC	dendritic cell
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAS	gamma-activated sequence
gDNA	genomic DNA
GrzB	granzyme B
GUSB	β-glucuronidase
HBSS	Hank’s balanced salt solution
HPRT	hypoxanthine phosphoribosyltransferase
IFN	interferon
IFNAR	IFNα receptor subunit
IFNGR	IFNγ receptor subunit
IκB	transcription factor inhibitor
IKK	IκB kinase
IL	interleukin

iNOS	nitric oxide synthase
IRAK	IL-1-receptor associated kinase
IRAK-M	IL-1 receptor-associated kinase M
IRF	IFN regulatory factor
IRM	immune response modifier
ISGF-3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response element
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
LRR	leucine rich repeat
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MKK	MAPK kinase
MyD88	myeloid differentiation primary response gene
MyD88s	spliced variant of MyD88
NADPH	nicotinamide adenine dinucleotide phosphate
NEMO	IKK subunit NF-κB essential modifier
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NOD	nucleotide-binding oligomerization daomain
NLR	NOD-like receptor
OD	optical density
ODN	oligodeoxynucleotide
OAS	2'-5'-olygoadenylate synthase
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
PIAS	proteininhibitor of activated Stat
PFU	plaque forming units
PKR	protein kinase
PO	phosphodiester
Poly I:C	polyinosinic polycytidylic acid
PRF	perforin
PRR	pattern recognition receptor
PTO	phosphorothioate
rfeIFN	recombinant feline interferon
rhuIFN	recombinant human interferon
RIG-I	retinoid acid-inducible gene I
RIP1	receptor-interacting protein 1
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute cell culture medium
RPS7	ribosomal protein S7
RT-PCR	reverse-transcriptase polymerase chain reaction
R-848	resiquimod
SARM	sterile-alpha and Armadillo motif containing protein

SHP-1	Src homology-2 domain–containing phosphatase-1
SOCS	suppressors of cytokine signaling
SPF	specified pathogen free
SSI	STAT-induced STAT inhibitors
ssRNA	single-stranded RNA
STAT	signal transducer and activator of transcription
TAB	TAK1-binding protein
TAK1	transforming growth factor-activated protein kinase 1
TANK	TRAF family member-associated NF-κB activator
TBK1	TANK binding kinase 1
Th cell	T helper cell
TIR	Toll/ Il-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF-receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain containing adaptor inducing IFNβ
VBS	vaccine breakdown strains
VPg	viral protein genome-linked
VSD	virulent systemic disease
VSV	vesicular stomatitis virus
YWHAZ	tyrosine 3-monooxygenase

1. Summary

CpG VR-1, a type A CpG ODN and TLR9 ligand, has been shown to induce effective production of type I IFN in mice. In this study, the potential of this molecule to stimulate the feline innate immune system and temporarily induce unspecific resistance to viral infections in vitro was tested.

In feline PBMCs, CpG VR-1 was able to highly up-regulate the gene expression of type I IFNs (IFNα, IFNβ, IFNω by mean factors of 1440, 394 and 1908, respectively) and Mx (49x), to induce the proinflammatory cytokines IL-6 (10x) and TNFα (2x) and enhance the expression of IL-12 (7.5x) and IL-15 (7.4x) as well as Granzyme B (2.5x). Also, stimulated PBMCs released soluble factors into the cell culture supernatant, among which biologically active type I IFNs. When CRFK and Fcwf-4 cells were incubated with these supernatants, not only Mx gene expression was highly induced but replication of FCV, FHV and VSV could also be inhibited in these cells. The expression factors of the Mx gene in the target cells correlated directly with those of the type I IFNs in the PBMCs. The survival of the Fcwf-4 cells upon viral challenge (FCV, FHV, or VSV) was correlated to their Mx gene expression induced by the treatment with the same supernatants. In CRFK cells, only the inhibition of VSV was correlated to the Mx gene expression. The potent inhibition of viral replication upon target cell treatment with the supernatants from PBMCs treated with CpG VR-1 represents promising results for further in vivo studies.

2. Introduction

Viral infections, particularly new emerging viruses represent a great threat worldwide in human and veterinary medicine. Vaccines which induce a specific long-term protection have been successfully used as prevention of numerous diseases. However, the development of effective and safe vaccines against new viruses or antigenetically variant strains requires an enormous amount of time in which the disease is unrestrictedly allowed to spread within a population. As an alternative to vaccination the stimulation of the innate immune system provides new possibilities in treatment and prevention.

In mammals, invading pathogens are counteracted by the innate immune system. This first line of defence is an extremely complicated system, including countless factors working together to induce an optimal and quick response. It not only recognizes pathogens and generates an immediate antimicrobial response, but also activates subsequent adaptive immune mechanisms, resulting finally in a long-lasting and specific answer.

The innate immune system contains pathogen recognition receptors (PRR) with which it recognizes highly conserved molecular structures in microbes called pathogen-associated molecular patterns (PAMPs). Viral nucleic acids, for example, are detected by different toll-like receptors (TLR3: dsRNA; TLR7/8: ssRNA; TLR9: CpG DNA) present in the membranes of endosomic compartments of various cell types, especially dendritic cells. Upon ligand binding, TLRs induce complex downstream signalling cascades activating interferon regulatory factors IRF3 and IRF7, NF-κB and other transcription factors leading to an enhanced expression of type I interferon and proinflammatory genes. IFNα, a type I IFN, is a potent antiviral agent. It stimulates in yet uninfected cells the production and activation of many proteins that interfere with viral replication such as PKR, OAS, ADAR1 and Mx, inducing an antiviral state. It also increases antigen presentation by inducing cell –surface expression of MHC class II molecules and stimulates macrophages, NK cells, T- and B-lymphocytes, linking the innate with the adaptive immune response.

Type I IFN is already established as successful in the treatment of many infectious diseases in humans and animals. In the cat, IFNω is used in the therapeutic protocols against important viral infections such as FIV, FeLV, FHV and FCV. Stimulating directly the innate immune system in a similar way as pathogens do and therefore inducing an even broader immune answer, could possibly have advantages in therapy and in studying the early immune mechanisms. Synthetic analogues of viral PAMPs called immune response modifiers (IRMs) to stimulate TLRs exist and have been studied intensively over the last years.

In this study we investigated the changes in the gene expression patterns of various cytokines, namely IFNα, IFNβ, IFNγ, IFNω, IL-4, IL-6, IL-10, IL-12, IL-15, TNFα, Perforin, GranzymeB and the IFN-inducible Mx in feline PBMCs after stimulation with CpG VR-1 (a TLR9 ligand) and the antiviral potential of the produced cell supernatant in vitro against FCV and FHV infections in feline cells.

3. Literature

3.1. Immunological aspects

3.1.1. Innate immunity

The innate immune system is the first line of defence against invading pathogens. It is principally based on physical and chemical barriers as well as on different cell types. In contrast to the adaptive immune system, it is relatively non-specific and is mediated mainly by phagocytic cells and antigen-presenting cells (APCs) such as granulocytes, macrophages, and dendritic cells (DCs). The ability to inhibit microbes during early infection is mostly achieved upon pathogen recognition by the activation of complement, phagocytosis, and other immune responses. Pattern recognition receptors (PRRs) such as the membrane bound Toll-like receptors (TLRs), the cytosolic PRRs, retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), recognize pathogens by structures, called pathogen-associated molecular patterns (PAMPs), which were highly conserved throughout evolution. These PAMPs are similar for whole classes of pathogens, are essential for their survival and differ in their molecular structures from those of the host. Some PRRs also recognize host factors when they are in atypical locations or have formed abnormal molecule complexes. Upon ligand binding, PRRs activate a multitude of intracellular signalling pathways, inducing an early innate host response and also providing a link to the adaptive immune response. (Mogensen 2009)

3.1.2. DCs

Dendritic cells (DCs) are present in the tissues of all organs as well as in the circulation and in lymphatic organs. They have a broad range of receptors (PRRs) recognizing viral molecular patterns. Triggering of these patterns initiates the innate antiviral immune response which is essential for limiting viral spreading. DCs produce inflammatory cytokines and antiviral interferons in response to and to combat viral infections and play an essential role in recognizing, processing and presenting antigen (Ag) thus linking innate and adaptive immunity (Eisenacher et al. 2007; Van de Walle et al. 2009).

3.1.2.1. DC subgroups

DCs are a diverse group of cells that can be differentiated based on their origin, localisation and function. There are two major subgroups, the conventional DCs (cDCs) and the plasmacytoid DCs (pDCs) (Van de Walle et al. 2009).

3.1.2.1.1. Conventional DC (cDCs)

Two types of cDC are described, first the migratory DCs which are present in great numbers in peripheral and lymphoid tissues (i.e. Langerhans cells) and in small amounts in the blood. These cDCs express several types of PRRs and they constantly phagocyte Ag. Upon activation, they migrate

to the draining lymph nodes, secrete IL-12 and mature into Ag-presenting cells able to activate naïve T cells. Secondly, there are non-migratory cDCs located in the lymphoid tissue collecting and presenting Ag there (Van de Walle et al. 2009).

3.1.2.1.2. Plasmacytoid DC (pDCs)

pDCs are also described as natural interferon producing cells, characterized by their ability to express large amounts of type I interferon upon activation (up to 1-2 IU per cell or 10-100 times more than other cell types) which makes them key effector cells in early antiviral innate immune response. They are mostly found in the blood and are present in tissues only in small numbers. Compared to cDCs they are poor in Ag-presentation. Besides type I IFN, TLR-activated pDCs also produce high levels of TNFα, IL-6, and several chemokines (Van de Walle et al. 2009).

3.1.2.2. DC ontogeny

The cDCs either arise from a common DC precursor (CDP) or from a macrophage DC precursor (MDP) which both develop from the myeloid precursors. cDCs leave the bone marrow as immature migratory cDCs and circulate or mature into lymphoid tissue-resident DCs. MDPs also give rise to monocytes which can further develop into migratory DCs themselves, macrophages or form inflammatory DCs during infection. pDCs develop also from CDP or from lymphoid precursors in the bone marrow and are already differentiated when they migrate to the blood (Figure 1) (Van de Walle et al. 2009).

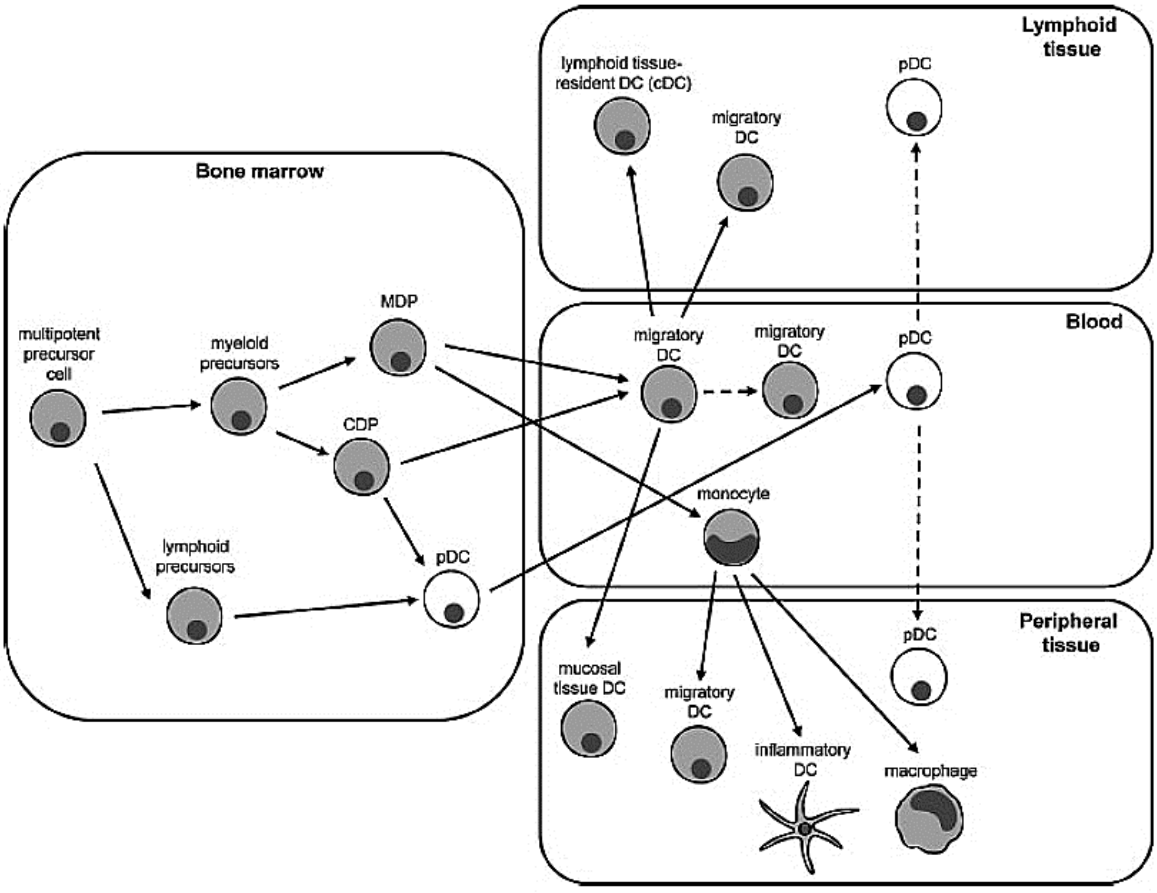

Figure 1: Development of DC subpopulations (Van de Walle et al. 2009)

3.1.2.3. DC activation and maturation

During viral mucosal or skin infection epithelial cells and tissue DCs are the first cells exposed to the invading virus. Epithelial cells secrete ligands (chemokines) that quickly attract non-activated blood DCs. Maturing DCs loose endocytic activity and express more MHC class I and II on their cell surface and the MHC-peptide complexes show an increased stability. There is also an upregulation of adhesion and co-stimulatory molecules. DCs undergo morphologic changes such as increasing motility and start to produce inflammatory cytokines and chemokines. A first wave of chemokines attracts T-helper (Th) 1 cells and neutrophils, a second wave memory T-cells and monocytes. cDCs migrate via the lymphatic circulation into the draining lymph nodes whereas pDCs enter the lymph nodes from the blood. In the lymph node DCs secrete a third wave of chemokines attracting naïve T- and B-cells as well as Th2 and regulatory T-cells. DCs induce a T-cell mediated immune response which regulates the immune effectors including Ag-specific CD8+ cytotoxic T-cells, antibody-producing B-cells, macrophages, eosinophils and NK cells (Van de Walle et al. 2009).

3.1.3. TLRs

3.1.3.1. Morphology

Toll-like receptors are transmembrane receptors expressed on numerous cell types including macrophages and dendritic cells. In humans a total of 10 TLRs are known and they all recognize distinct PAMPs from microbial pathogens including viruses, bacteria, fungi and protozoa (Table 1). On the extracellular or luminal side, these glycoproteins contain leucine-rich repeat (LRR) motifs which are involved in ligand recognition and signal transduction. In the cytoplasmic compartment they have a Toll/IL-1 receptor (TIR) domain required for intracellular signalling (Carpenter et al. 2007; Mogensen 2009).

3.1.3.2. Localization

The TLR family can be divided into two subgroups, one of which is expressed on the cell surface (TLR1, -2, -4, -5, -6, -10) recognizing bacterial and fungal cell wall components as well as some viral proteins, and the other (TLR3, -7, -8, -9) with their receptors localized in the membranes of intracellular compartments such as endosomes and lysosomes, detecting microbial nucleic acids, particularly viral DNA and RNA (Eisenacher et al. 2007; Mogensen 2009).

3.1.3.3. Ligands

TLR2 is able to recognize a large spectrum of microbes by responding to lipoproteins, lipopeptides, peptidoglycans and various other microbial structures. In a heterodimer with TLR1 (TLR1/TLR2), it responds to triacylated lipoproteins and in one with TLR6 (TLR2/TLR6) to diacylated lipopeptides. TLR4 recognizes lipopolysaccharide, the major component of the cell walls of Gram-negative bacteria, and also endogenous ligands such as heat shock proteins if presented in high concentrations.TLR5 is localized on the basolateral side of intestinal epithelial cells and recognizes flagellin, a monomeric component of bacterial flagella, from bacteria that have entered the mucosa. (Takeda et al. 2005; Carpenter et al. 2007)

TLR3 recognizes double-stranded RNA (dsRNA) which is produced by many viruses during replication. TLR7 and TLR8 are structurally quite similar and both recognize guanosine- or uridine-rich single-stranded RNA (ssRNA). ssRNA is abundant in host cells however host-derived ssRNA is not detected since it is not delivered to the endosomes. They also respond to a large number of synthetic antiviral components for example imidazoquinolines. The ligand for TLR9 is unmethylated CpG DNA which is present in the genomes of both viruses and bacteria. It can also recognize synthetic oligodesoxyribonucleotides (ODN) containing unmethylated CpG sequence motifs. In vertebrates, CpG motifs are highly reduced and their cysteines are methylated. (Takeda et al. 2005; Carpenter et al. 2007)

TLR10 can form heterodimers with TLR1 or TLR2, but its ligand or function is still unknown (Dasari et al. 2008). TLR11 is only functional in mice and plays a role in the defense against uropathogenic E.coli (Carpenter et al. 2007).

There are also several TLR-independent pathogen recognition-pathways. Table 1 provides more detailed information (Mogensen 2009).

Table 1 : Recognition of microbial components by PRRs (Mogensen 2009)

Receptor	Cellular localization	Microbial component(s)	Origin(s)
TLRs			
TLR1/TLR2	Cell surface	Triacyl lipopeptides	Bacteria
TLR2/TLR6	Cell surface	Diacyl lipopeptides	*Mycoplasma*
		Lipoteichoic acid	Gram-positive bacteria
TLR2	Cell surface	Lipoproteins	Various pathogens
		Peptidoglycan	Gram-positive and -negative bacteria
		Lipoarabinomannan	Mycobacteria
		Porins	*Neisseria*
		Envelope glycoproteins	Viruses (e.g., measles virus, HSV, cytomegalovirus)
		GPI-mucin	Protozoa
		Phospholipomannan	*Candida*
		Zymosan	Fungi
		β-Glycan	Fungi
TLR3	Cell surface/endosomes	dsRNA	Viruses
TLR4	Cell surface	LPS	Gram-negative bacteria
		Envelope glycoproteins	Viruses (e.g., RSV)
		Glycoinositolphospholipids	Protozoa
		Mannan	*Candida*
		HSP70	Host
TLR5	Cell surface	Flagellin	Flagellated bacteria
TLR7/8	Endosome	ssRNA	RNA viruses
TLR9	Endosome	CpG DNA	Viruses, bacteria, protozoa
RLRs			
RIG-I	Cytoplasm	dsRNA (short), 5′-triphosphate RNA	Viruses (e.g., influenza A virus, HCV, RSV)
MDA5	Cytoplasm	dsRNA (long)	Viruses (picorna- and noroviruses)
NLRs			
NOD1	Cytoplasm	Diaminopimelic acid	Gram-negative bacteria
NOD2	Cytoplasm	MDP	Gram-positive and -negative bacteria
NALP1	Cytoplasm	MDP	Gram-positive and -negative bacteria
NALP3	Cytoplasm	ATP, uric acid crystals, RNA, DNA, MDP	Viruses, bacteria, and host
Miscellaneous			
DAI	Cytoplasm	DNA	DNA viruses, intracellular bacteria
AIM2	Cytoplasm	DNA	DNA viruses
PKR	Cytoplasm	dsRNA, 5′-triphosphate RNA	Viruses

3.1.3.4. Signaling

TLR-induced downstream signaling can be differentiated by the adaptor molecules, i.e. MyD88-dependent or MyD88-independent TRIF-dependent pathway. Signaling is mediated by phosphorylation, ubiquitination, protein-protein interactions and finally activation of transcription factors. Nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs) are capable of inducing a proinflammatory response and IFN regulatory factors (IRFs) are essential for stimulation of IFN production. (Mogensen 2009)

3.1.3.4.1. MyD88-dependent signaling pathway (Figure 2)

MyD88-dependent signaling is activated by all TLRs except TLR3. After ligand binding TLRs dimerize and undergo conformational changes. MyD88 protein associates with the cytoplasmic part of the receptor and recruits members of the IL-1 receptor (IL-1R)-associated kinase (IRAK) family. IRAK4, IRAK1/2 are sequentially phosphorylated and IRAK1 or alternatively IRAK2 associate with TNF-receptor-associated factor 6 (TRAF6) which is an ubiquitin protein ligase. It adds, together with the ubiquitination enzyme complex, K63-linked polyubiquitin chains to TRAF6 itself and other molecules including the transforming growth factor-activated protein kinase 1 (TAK1) and IκB kinase (IKK) subunit NF-κB essential modifier (NEMO). TAK1-binding protein 2 (TAB2) and 3 (TAB3) associate with the ubiquinated TRAF6 bringing TAK1 close to the signaling complex which leads to its activation. TAK1 then stimulates two distinct signaling pathways, one involving the IKK complex and the other the MAPK pathway (Mogensen 2009).

In the first pathway TAK1 activates the IKK complex, which is an essential part of many inflammatory signaling pathways and is composed of the two kinases IKKα and IKKβ as well as the chaperone IKK complex protein and the adaptor NEMO/IKKγ, by phosphorylation of the IκB protein. IκB then undergoes proteasomal degradation to allow activation and translocation of NF-κB to the nucleus, where it binds to κB-binding sites in promoters and enhancers of proinflammatory genes which are then transcribed (Mogensen 2009).

In the second pathway, TAK1 phosphorylates members of the MAPK kinase (MKK) family which then activate either p38 or c-Jun N-terminal kinase (JNK) leading to the activation of the transcription factor activator protein 1 (AP1) (Mogensen 2009).

Additionally to the MyD88-dependent induction of proinflammatory cytokines, TLR7/8 and TLR9 can also stimulate IFN type I gene expression in a pathway requiring MyD88 encoded protein in contrast to IFN-stimulation by TLR3 and TLR4, which is dependent on TRIF. In pDCs TLR7/8- and TLR9-signaling results in the formation of a complex of MyD88 protein, TRAF6 and IRF7 activating IRF7 and thus the production of type I IFN (Kawai et al. 2004).

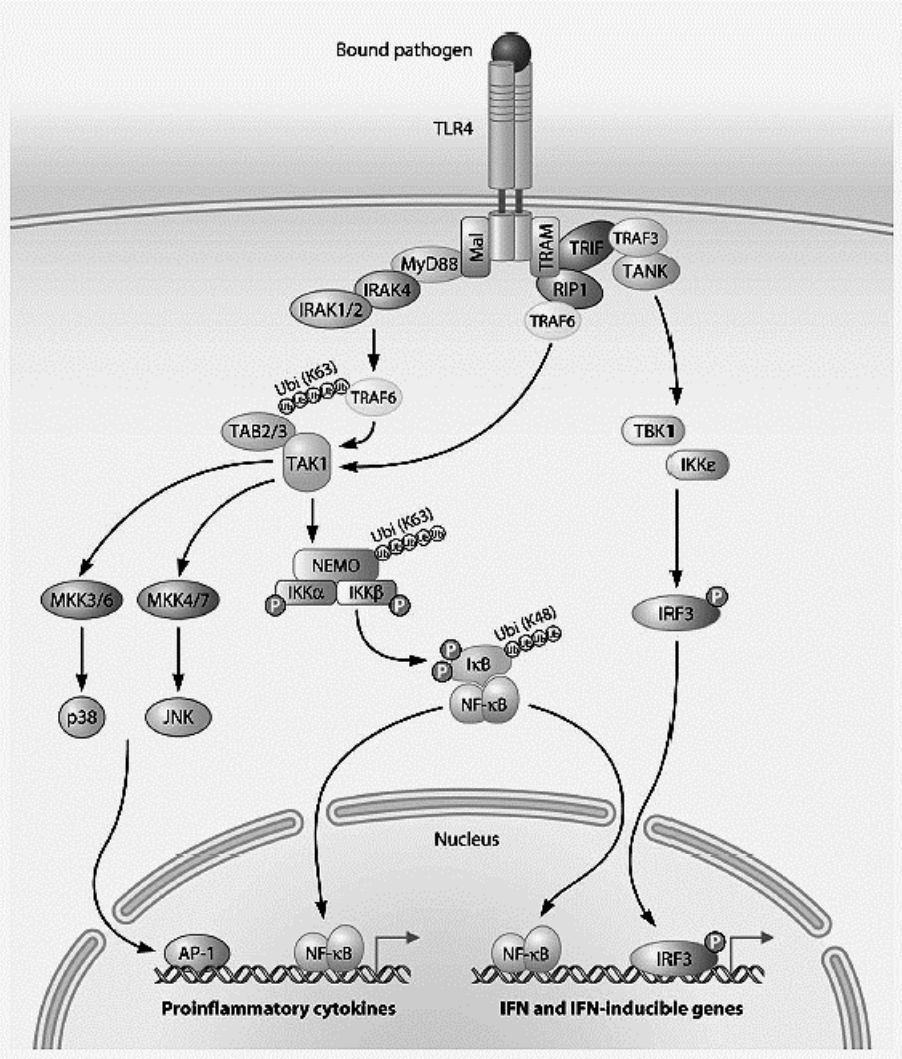

Figure 2: MyD88-dependent signaling pathway (Mogensen 2009)

3.1.3.4.2. MyD88-independent, TRIF-dependent signaling pathway (Figure 2)

A MyD88-independent signaling pathway is used by TLR3 and also by TLR4 additionally to the MyD88-dependent signaling. The TIR-domain containing adaptor inducing IFNβ (TRIF) associates in the case of TLR4 with the TRIF-related adaptor molecule (TRAM) and in the case of TLR3 directly with the TIR domain. The interaction of TRIF with TRAF3 and TANK leads to complex formation with the IKK-related TBK1 and IKKε. These then phosphorylate the interferon regulatory factors 3 and 7 (IRF3 and 7). IRF3 and 7 form hetero- or homodimers, translocate to the nucleus and bind, together with transcriptional coactivators, to target sequences in DNA such as IFN-stimulated response elements (ISRE). IRFs, NF-κB and AP1 form a multiprotein complex called the enhanceosome which induces transcription of IFNβ gene. TRIF can also contribute to the activation of NF-κB by binding TRAF6 and the receptor-interacting protein 1 (RIP1) resulting in subsequent activation of TAK1 and the two downstream pathways of IKK- or MAPK-signaling (Eisenacher et al. 2007; Mogensen 2009).

3.1.4. NF-κB inducible proinflammatory mediators

NF-κB plays a central role in both innate and adaptive inflammation and immunity by inducing many genes including those for proinflammatory cytokines such as IL-1, IL-6 and TNFα as well as

chemokines and by upregulating cell adhesion molecules and immunoreceptors, i.e. cytokine and chemokine receptors, immunoglobulins, TLRs, MHC molecules, and costimulatory molecules. Together, these NF-κB inducible proteins recruit leukocytes to sites of inflammation, enhance phagocytosis and antigen presentation and participate in complement- or NK cell-mediated cellular lysis (Mogensen 2009).

3.1.5. Interferon

Type I IFN, playing a major role in innate antiviral response, includes of IFNα, IFNβ and IFNω and is induced by PRR signaling in most cell types. IFNγ on the other hand is a type II IFN, produced by T lymphocytes and NK cells and is important for macrophage activation. IFNλ is a type III IFN. pDC produce the majority of type I IFN during viral infection. In these cells TLR7/8 and TLR9 induce type I IFN in a MyD88-dependent manner. In macrophages, cDC and fibroblasts, type I IFN is induced by cytoplasmic RNA via RLR-mediated signaling and by TLR3 and TLR4 in a TRIF-dependent pathway (Mogensen 2009).

3.1.5.1. Positive feedback mechanism

There is a positive feedback mechanism in IFN regulation increasing IFN response. IRF3 is constitutively expressed whereas the expression of IRF7 is low in unstimulated cells, except in pDCs. Early IFNβ is therefore mostly dependent on IRF3 signaling, secreted IFNβ then acts on the surrounding cells and stimulates IRF7 expression thus enhancing the signaling response and amplifying the production of IFNα and IFNβ (Mogensen 2009).

3.1.5.2. Induction of IFN-inducible genes (Figure 3)

Type I IFNs signal through a cell surface receptor consisting of two subunits IFNAR-1 and IFNAR-2. IFNγ binds to a different receptor with the subunits IFNGR-1 and IFNGR-2. IFN mediates the heterodimerization of the receptor subunits. Receptor-associated Janus kinases (JAKs) are activated when IFN binds to the receptor and phosphorylate latent cytoplasmic signal transducers and activators of transcription (STATs). There are four members of the JAK family (Jak-1, Jak-2, Jak-3, Tyk-2) and seven of the STAT protein family (Stat-1, -2, -4, -5a, -5b, 6). Jak-1 and Tyk-2 bind to the IFNα/β receptor and Jak-1 and Jak-2 to the IFNγ receptor. Phosphorylated Stat-1α/β and Stat-2 of the IFNα/β signaling pathway translocate with the protein p48 (IRF9) to the nucleus and form a complex called IFN-stimulated gene factor 3 (ISGF-3) which binds to cis-acting DNA elements (ISRE) in IFNα/β-inducible genes. For IFNγ, phosphorylated Stat-1α homodimerizes, translocates and binds to other cis-acting elements, the gamma-activated sequence (GAS) of IFNγ-inducible genes, resulting in the expression of IFN-stimulated genes. (Samuel 2001)

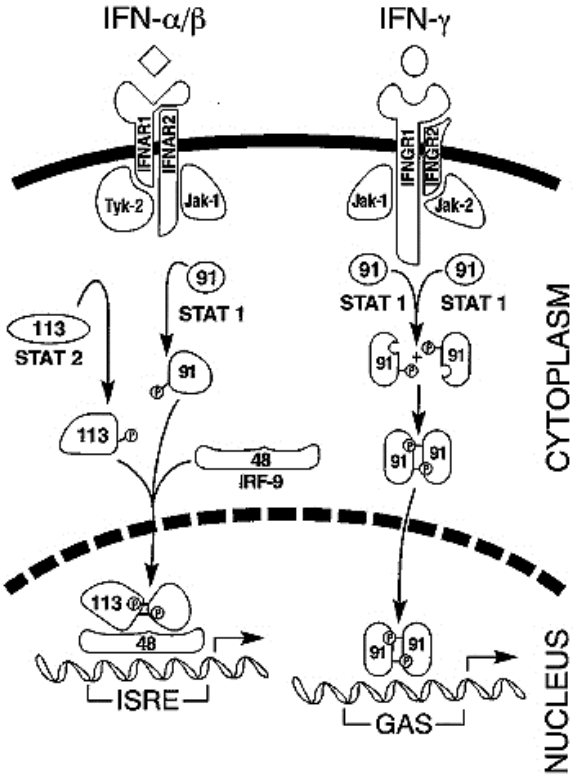

Figure 3: JAK-STAT signaling pathway (Samuel 2001)

3.1.5.3. Negative Regulators

To prevent inappropriate and excessive inflammation, which may be harmful to the host, innate immunity requires cellular negative regulators to be able to control and down-regulate the inflammatory response. Pathogens themselves have developed during evolution, various strategies to interfere in pathogen recognition and signalling (Mogensen 2009).

3.1.5.3.1. Cellular negative signalling

The TLR adaptor molecule SARM acts as a negative regulator of TRIF-dependent signalling from TLR3 and TLR4. Another inhibitory TLR adaptor molecule is the protein encoded by MyD88s, a spliced variant of MyD88, which does not bind to IRAK4. IRAK-M belongs to the IRAK family but has no kinase activity, preventing downstream signalling. The enzymes, A20 and CYLD, deubiquitinize TRAF6, thereby regulating TLR- and TNFα-induced NF-κB activation. TRIM30α interferes with NF-κB activation by degrading TAB2/3 which is required for TAK1 activation. Since TRIM30α itself is activated by NF-κB, this presents a negative feedback loop. NF-κB also induces gene expression of new IκB that binds to free NF-κB preventing it from further stimulating inflammatory genes. IKKα degrades NF-κB subunits bound to DNA terminating the promotion. The suppressors of cytokine signalling (SOCS) are induced by many cytokines, including IFNγ. SOCS-1 suppresses the activation of all four JAKs and therefore inhibits both, IFNα/β and IFNγ signalling. Further cellular negative feedback regulators are STAT-induced STAT inhibitors (SSI) and cytokine-inducible SH2 protein (CIS). Protein inhibitor of activated Stat (PIAS) 1 and 3 interfere in Stat-signalling by interacting with the Stat-1 dimer blocking its DNA-binding activity. The protein tyrosine phosphatase SHP-1 interacts

with JAKs and catalyzes their dephosphorylation. Additionally, there are also inhibitors of cytosolic PRR signalling (Samuel 2001; Mogensen 2009).

3.1.5.3.2. Viral antagonists

Viruses have developed many different mechanisms to inhibit the induction of proinflammatory molecules and IFN. Some viruses encode for products that imitate cellular components and thus lead to an antagonism, for example by producing a soluble IFN receptor homologue (poxviruses) which binds and blocks IFN or viral proteins with TIR-like domains competing to bind MyD88 proteins and TRIF (vaccinia virus) and human herpesvirus 8 synthesizes an IRF homologue. Vesicular stomatitis virus protein M inhibits the export of mRNAs, including those of IFNα/β, from the nucleus to the cytoplasm. Other viruses block IFN-mediated signaling (adenovirus' protein E1A binds a coactivator of transcription), interfere with the transcriptional activation of IFN-inducible cellular genes (paramyxovirus) or inhibit the action of IFN-inducible proteins for example by binding activator RNAs. Some viruses, on the other hand, stimulate NF-κB activation because it increases viral replication in viruses with NF-κB DNA-binding sites in their promoters (HIV) and oncogenic viruses modulate cellular growth and apoptosis promoting malignant transformations (Samuel 2001; Mogensen 2009).

3.1.5.4. IFN-induced proteins and their antiviral activities (Figure 4)

Antiviral activities induced by type I IFN are mediated by protein kinase (PKR), 2'-5'-olygoadenylate synthase (OAS) and RNase L, RNA-specific adenosine deaminase (ADAR) and Mx proteins. IFN also induces a form of nitric oxide synthase (iNOS2) and the MHC class I and II molecules (Samuel 2001).

Furthermore, type I IFN plays a role in the inhibition of cellular growth and control of apoptosis however it may be pro- or antiapoptotic depending on the cellular situation. It also participates in adaptive immune defences such as cross-presentation of viral antigen, B-lymphocyte isotype switching and differentiation into plasma cells and DC maturation. IFNγ activates macrophages and intracellular killing of microbial pathogens and cooperates with type I IFN in many aspects of innate and adaptive immunity (Mogensen 2009).

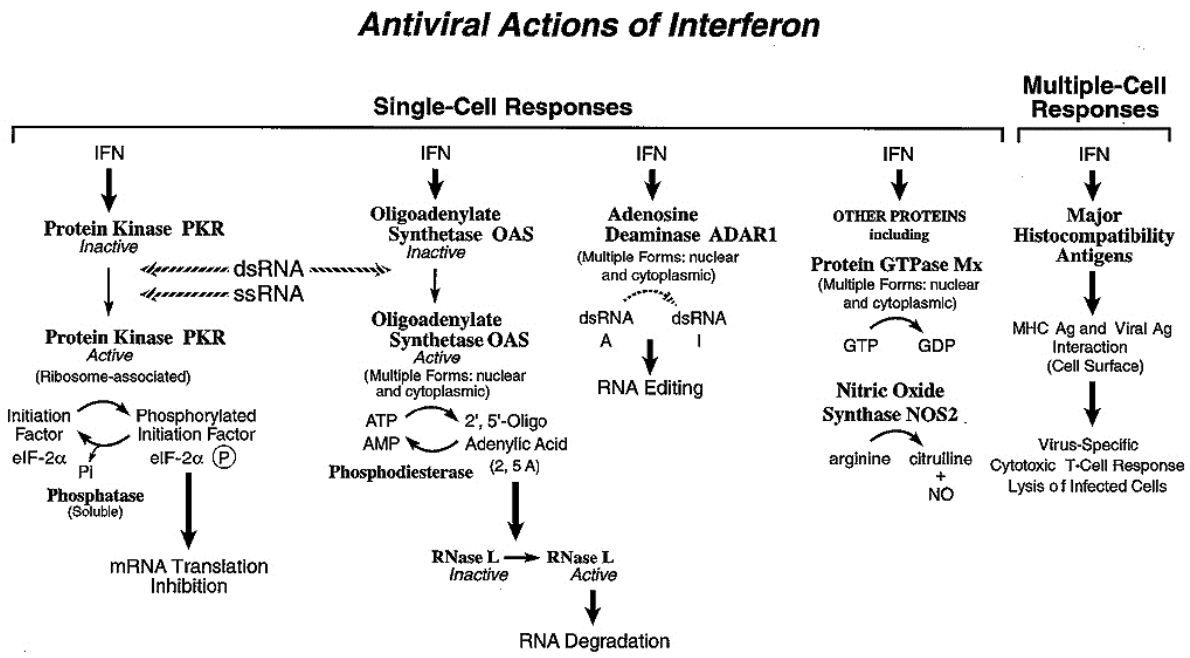

Figure 4: Antiviral actions of interferon (Samuel 2001)

3.1.5.4.1. PKR

The IFNα-induced PKR is a RNA-dependent protein kinase mainly found in the cytoplasm and associated with ribosomes. PKR is activated by autophosphorylation mediated by dsRNA. Upon activation it phosphorylizes proteins such as the α subunit of protein synthesis initiation factor 2 (eIF-2α) or the transcription factor inhibitor IκB. eIF-2α normally catalyzes guanine nucleotide exchange, however this reaction is impaired by the phosphorylation which leads to an inhibition of translation thus playing an important role in antiviral action as well as in control of cell growth mediated by IFNs (Samuel 2001).

3.1.5.4.2. 2'-5'-olygoadenylate synthase (OAS) and RNase L

2'-5'-olygoadenylate synthase (OAS) and RNase L are two enzymes, induced by IFNα,-β or -γ and activated by dsRNA, leading together to RNA degradation. There are three forms of OAS (OAS1, -2, -3) in humans differing in size, localization (membranes, cytoplasm, nucleus), amount of dsRNA required for their activation and optimal conditions for enzyme activity. They catalyze the synthesis of oligoadenylates with different pattern sizes, which contain a 2',5'-phosphodiester bond linkage. By binding to 2'-5'A oligonucleotides, the latent monomeric endoribonuclease RNase L converts to the active homodimeric form. Activated RNase L degrades both viral and cellular RNAs including cellular rRNA, by cleaving on the 3' side of UpXp-sequences (Samuel 2001).

3.1.5.4.3. RNA-specific adenosine deaminase ADAR1

The IFN-inducible RNA-specific adenosine deaminase ADAR1 is an important RNA-editing enzyme of viral RNA transcripts and cellular pre-mRNAs. It catalyzes the deamination of adenosine to inosine destabilizing the dsRNA helix. The RNA becomes more single stranded because IU base pairs are less stable than AU pairs. The A-to-I transformation is of major biological importance, because inosine

is recognized as guanine by the polymerases and ribosomes resulting in changes in protein-coding and the sequence of replicated RNAs (Samuel 2001).

3.1.5.4.4. Protein Mx GTPase

Proteins of the Mx family are antiviral molecules with GTPase activity. They associate with themselves or with viral protein complexes. Mx is induced by IFNα and IFNβ but not IFNγ. Mx proteins vary in their antiviral activities and molecular mechanisms by which they inhibit viral replication depending on the specific Mx protein, its subcellular localization and the type of challenging virus. Mx present in the cytoplasm can inhibit viral nucleocapsid transportation or block RNA synthesis, when present in the nucleus. Cells that constitutively express the human MxA protein show a high degree of antiviral activity and resistance to several viruses (Samuel 2001). Mx is a potent antiviral protein which can even induce sufficient protection against viruses in the absence of other IFNα/β-inducible proteins (Hefti et al. 1999; Samuel 2001).

3.1.5.4.5. Major histocompatibility complex proteins

Besides stimulating antiviral effects in infected cells, IFNα/β and IFNγ both have immunoregulatory functions modulating interactions of cells, for example NK cells or Th cells, with virus-infected cells. IFNα/β and IFNγ lead to increased levels of class I MHC molecules but only IFNγ induces MHC class II molecules efficiently by stimulating the expression of the MHC class II transactivator factor (CIITA). IFNγ also modulates the expression of cellular components of the proteasomes thus affecting the processing and presentation of antigenetic peptides through MHC class I molecules (Samuel 2001).

3.1.5.4.6. Inducible nitric oxide synthase

iNOS sequence contains a GAS element in its promotor and is therefore induced by IFNγ. It oxidizes, NADPH-dependently, L-arginine to nitric oxide (NO) and citrulline. NO is an antimicrobial and antiviral agent playing a role in immunological defences. It can be changed by oxidation, reduction or adduction to other reactive nitrogen products which for example mediate the killing of infected cells. The cytotoxic effects are induced by reacting with iron in mitochondrial enzymes thus affecting electron transportation or cellular regulatory proteins (Samuel 2001).

3.1.5.5. Interferons used in the cat

The amino acid sequence of rfeIFN has about 60% homology to huIFNα1 (Nakamura et al. 1992). Cats develop neutralizing antibodies against rhuIFNα which shortens the efficacy in long term therapy (Zeidner et al. 1990). Species-specific feline IFNα (feIFNα) is therefore better especially for the treatment of chronic viral infections in cats (Baldwin et al. 2004). In humans, 13 IFNα subtypes exist. In cats five subtypes (IFNα-1, IFNα-2, IFNα-3, IFNα-5, IFNα-6), sharing 95-99% sequence homology, have been described, cloned and tested for their antiviral activities. IFNω (available for treatment of the cat as Virbagen® Omega) is distinguished from IFNα by an extension of six amino acid codons at the C-terminus of IFNω and the number and alignment of proline residues. IFNα-1, IFNα-2, IFNα-5, IFNα-6 showed a significant reduction in VSV induced cytopathic effect in CRFK cells, whereas IFNα-3 had an impaired antiviral activity (Wonderling et al. 2002).

3.1.5.6. Inhibitory effects of interferon on feline herpes- and calicivirus

Mochizuki et al. could show a reduction of FHV yield in fcwf-4 cells of 1.5 and 2.7 log10 upon treatment with 10 and 1'000U rfeIFNα, respectively. There was no further effect when the dose was raised to 10'000U. FCV was reduced dose-dependently from 0.8 to 3.5 log10 PFU in fcwf-4 cells and 0.7 to 1.8 log10 in CRFK cells. Continuous treatment induced a greater reduction in FCV yield than only pre-treatment. They compared the reaction of the different feline viruses upon IFN treatment to that of vesicular stomatitis virus (VSV), which is known to be extremely sensitive to IFN. VSV did not grow at all in fcwf-4 treated with only 10U. CRFK cells required slightly more IFN to be protected against VSV (Mochizuki et al. 1994).

3.1.6. Cytokines

3.1.6.1. IL-4

IL-4 is produced by Th2 CD4+ cells, basophils, mast cells, esosinophils, and NK cells. It plays a role in the differentiation of antigen-stimulated naïve T-cells into Th2 cells that produce IL-4 themselves as well as other cytokines including IL-5, IL-10 and IL-13. IL-4 suppresses IFNγ-producing CD4+ Th1 cells. In B cells IL-4 induces an antibody class switch toward the expression of IgE and IgG4 in humans and IgE and IgG1 in mice (Nelms et al. 1999).

3.1.6.2. IL-6

IL-6 has proinflammatory as well as anti-inflammatory cytokine functions. It plays for example an important role in inducing acute phase reactions and controlling the acute inflammatory responses by down regulating proinflammatory cytokines and up regulating anti-inflammatory molecules (Xing et al. 1998). IL-6 is on the other hand essential for antibody production by stimulating B cells to differentiate into antibody producing plasma cells. It also induces T-cell activation, growth and differentiation, stimulates macrophage development and acts as a growth factor for many cell types including the haematopoietic stem cells (Hirano 1998; Ding et al. 2009).

3.1.6.3. IL-10

The predominant effect of IL-10 is to reduce inflammation. It suppresses the maturation of APCs and their production of proinflammatory cytokines. By inhibiting APC maturation it prolongs their capability for antigen uptake and postpones the migration to the draining lymph nodes. On the other hand, IL-10 is a potent activator of NK cells and facilitates the target-cell destruction (Mocellin et al. 2003).

3.1.6.4. IL-12

IL-12 is synthesized by APCs such as macrophages, dendritic cells, B-lymphocytes, and astrocytes. It stimulates NK cell activity, induces IFNγ production by NK, Th1 and CD8 cells, regulates T-cell proliferation and differentiation of precursor T cells into Th1 cells and enhances CD8 CTL responses (Komatsu et al. 1998).

3.1.6.5. IL-15

IL-15 is produced by macrophages and other cell types in response to infectious agents. It controls growth and differentiation of T and B lymphocytes, enhances phagocytosis by neutrophils and macrophages and reduces their apoptosis. It stimulates the development and activation of NK cells and induces, in synergy with IL-12, IFNγ production by NK cells (Bannwart et al. 2007).

3.1.6.6. TNF alpha

TNFα is a proinflammatory cytokine mostly produced by macrophages but also by other cells including activated T cells, NK cells, Langerhans' cells, dermal mast cells and keratinocytes. Its production is stimulated by NFκB. Since almost every cell type has TNFα receptors, they are all affected by TNFα signaling and broad biological activities include cytotoxicity, cell differentiation, mitogenesis, and immunomodulation. Its main role in the acute inflammatory response is to stimulate the recruitment and activation of neutrophils and monocytes to the sites of infection. It increases the production of NFκB, IL-6, leukotriene and prostaglandin (LaDuca et al. 2001).

3.1.6.7. Perforin and Granzyme B

The pore-forming protein perforin (PRF) and the proapoptotic serine protease granzyme B (GrzB) are stored within secretory granules of NK cells and CTL. PRF forms pores into the target cell membrane or into the membranes of endocytic vesicles allowing Grz to pass through the pores into the cytosol where it cleaves endogenous or viral intracellular substrates leading to apoptotic cell death or viral inhibition (Hoves et al. 2010).

3.1.7. CpG

The endosomal TLR9 recognizes conserved sequences containing dinucleotides of unmethylated cytosine and guanine with a phosphodiester bond (CpG) (Heeg et al. 2008). In prokaryotic DNA sequences, these dinucleotides are found in the expected ratio of 1:16, compared to eukaryotic DNA in which these motifs are far less frequent. This is explained by the fact that cytosines are highly methylated within eukaryotic DNA and mCpG tends to mutate abnormally frequently to TpG (Bird 1980). Unmethylated CpG motifs thus are 100-fold more frequent in microbial DNA representing non-host DNA (PAMP) (Dalpke et al. 2004; Heeg et al. 2008).

3.1.7.1. Structure and modifications of synthetic CpG ODNs

TLR9 recognizes not only bacterial and viral CpG DNA but also short synthetic CpG oligonucleotides (ODN) (Heeg et al. 2008). They have to be at least about eight bases and include a CpG motif. The essential central CG is surrounded by flanking bases. An inversion of CG- to GC-sequences totally blocked immunostimulatory effects. The optimal sequences differ between humans and mice which indicates a degree of species specificity (Dalpke et al. 2004). GACGTT proved to be the best stimulatory sequence in mice, whereas GTCGTT was optimal for human TLR9 (Bauer et al. 2001).

The natural phosphodiester (PO) bonds in CpG DNA are rapidly degraded making synthetic PO CpG ODNs relatively inefficient. The modification with a complete phosphorothioate (PTO) backbone increases their stability against nucleases. These modifications however may induce effects on B cell

proliferation, Ab production, IL-6 secretion and macrophage chemotaxis that are not related to TLR9-signaling and induce in vivo splenomegaly and infiltration of tissues with mononuclear cells (Dalpke et al. 2004; Roberts et al. 2005).

Initially it was thought that CpG-ODNs have to be single-stranded for immunostimulation. However, later evidence showed that there occurs partial intermolecular duplex formation with single-stranded overhangs. The CpG sequence as well as the free 5' end were important for recognition, since completely double stranded molecules were inactive (Dalpke et al. 2004; Heeg et al. 2008).

3.1.7.2. Signalling

3.1.7.2.1. Uptake

CpG signalling is a two-step process. DNA uptake into endosomes is a process, mediated by multiple receptors, not depending on the CpG motifs, but rather influenced by backbone modifications and the flanking sequences. PTO backbones enhanced the uptake, as did the addition of poly-G nucleotides to PO-CpG-ODNs (Heeg et al. 2008). Poly-G strings only work when added at the 3' end and they did not work with PTO-CpG-ODNs at all. Formed endosomes undergo maturation and TLR9 molecules are delivered to the late endosome from the ER enabling recognition of the CpG motifs (Dalpke et al. 2004).

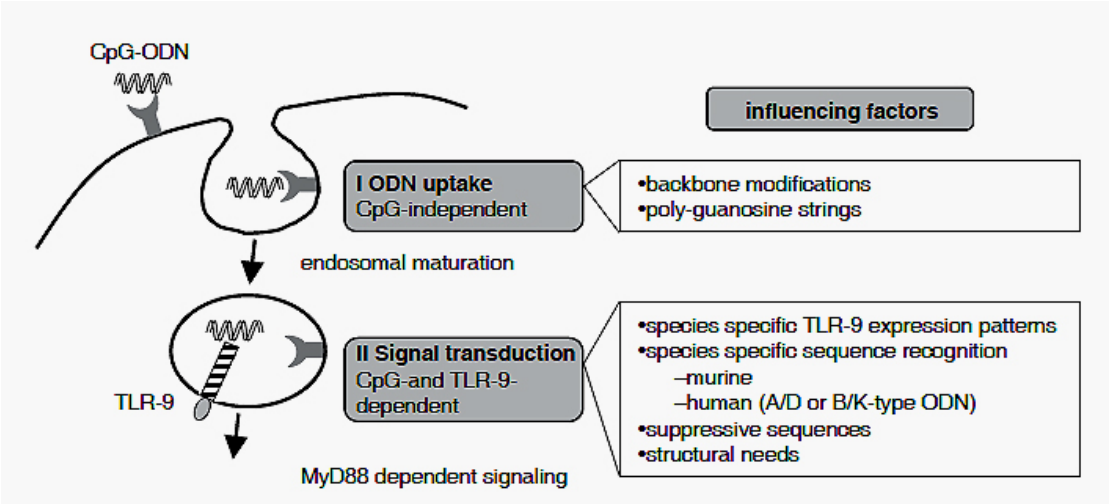

Figure 5: Two-step process of CpG-DNA signaling and influencing factors (Dalpke et al. 2004)

3.1.7.2.2. Signal transduction

TLR activation induces the classical TLR9 signaling cascade (see 3.1.3.4.1) resulting in proinflammatory response and the production of type I IFN. CpG showed a high capacity to induce IL-12 thus enhancing TH1-directed immune responses.

3.1.7.2.3. Classification

Different ODNs induce different immune response profiles, especially in human PBMCs. This was used to classify ODNs into subtypes, termed A/D-, B/K- or C- type CpG DNA. B/K-type ODNs are completely PTO modified and contain multiple TCG repeats. They induce B-cell proliferation and IL-6 secretion and are potent inducers of inflammatory cytokines such as IL-12 and TNF-a. A/D-type CpG

ODNs have a mixed backbone, possess a central Pu-Py-C-G-Pu-Py motif within flanking palindromic sequences and a poly-G tail at the 3' end. They induce high amounts of type I IFNs in pDCs stimulating NK cells to produce IFNγ, but have a reduced ability to induce IL-12. C-type ODNs have A/D as well as B/K activity (Dalpke et al. 2004; Takeda et al. 2005).

3.1.7.3. Therapeutic usage of CpG ODNs

As CpG ODN can nowadays be synthesized and modified easily, their broad range of immunstimulatory effects made them promising molecules in various therapies for example as immune response modifier during viral infections, as adjuvants in vaccinations, in cancer therapy and also in allergy treatment (Dalpke et al. 2004).

3.2. Feline herpesvirus

3.2.1. Taxonomy

Feline herpesvirus 1 is a member of the Varicellovirus of the Herpesvirus subfamily Alphaherpesvirinae. All isolates belong antigenetically to the same serotype and there are only minor genetic differences among strains (Gaskell et al. 2007).

3.2.2. Genome

The FHV genome consists of a linear double-stranded DNA which contains 134 kbp. It encodes for various enzymes and factors involved in the nucleic acid metabolism (DNA polymerase, helicase, primase, thymidine kinase etc.) (Roizmann et al. 1992).

3.2.3. Structure

The genome is packed within an icosahedral capsid, which is surrounded by a lipid bilayer envelope with embedded glycosylated proteins (McGeoch et al. 2006).

3.2.4. Epidemiology

The main host for FHV is the domestic cat, although virus could be isolated also from other felids such as cheetahs and lions and antibodies have been detected also in pumas. There is no evidence of human infection (Thiry et al. 2009).

FHV is spread worldwide in the cat population with viral prevalence, depending on the study, ranging from 1% (evaluated by virus isolation from oropharyngeal swabs) in healthy small populations (Binns et al. 2000) up to 18.3% in multiple cat households with respiratory problems (measured with multiplex real-time PCR in genomic DNA extracted from oropharyngeal and conjunctival swabs) (Gaston et al. 2004).

The two main sources of infection are first cats with primary infections and upper respiratory tract disease, which shed large amounts of virus particles and are therefore likely to transmit the disease, and second latently infected cats, in which intermittent reactivation gives rise to viral shedding (Stiles 2003; Thiry et al. 2009). Transplacental infection has not been demonstrated under natural conditions (Gaskell et al. 2007; Thiry et al. 2009).

Contamination of the environment is not an important source of infection except in catteries, because FHV is an enveloped virus and thus relatively fragile and short-lived in the environment. It only survives up to 18h in damp environment and less in dry conditions (Stiles 2003; Gaskell et al. 2007; Thiry et al. 2009). Moreover, FHV is highly susceptible to the effects of common disinfectants (Eleraky et al. 2002).

3.2.5. Pathogenesis

The virus enters via the nasal, oral or conjunctival routes. Primary sites of viral replication are epithelial tissues such as the mucosae of the nasal septum, turbinates, nasopharynx, and the tonsils. Conjunctivae, mandibular lymph nodes, and upper trachea are also often involved. Viraemia occurs rarely and has only been observed in neonates and hypothermic kittens or debilitated animals (Stiles 2003; Gaskell et al. 2007; Thiry et al. 2009). FHV causes a cytopathic effect and leads to multifocal epithelial necrosis with neutrophilic infiltration and inflammation. Recovery from acute symptoms takes about 10-14 days, however some animals may develop chronic lesions in the upper respiratory tract and ocular tissues. Viral excretion starts 24h after infection and generally persists for 1-3 weeks (Gaskell et al. 2007; Thiry et al. 2009).

As with other alphaherpesviruses, latency develops after the virus spreads along the sensory nerves and reaches neurons, especially the trigeminal ganglia, the main site of latency. Almost all infected cats become lifelong carriers, according to a study in 1977 at least 82% (Gaskell et al. 1977). Periodic viral reactivation, sometimes combined with recrudescence of mild clinical signs can occur particularly after periods of immunosupression induced by glucocorticoid treatment (reactivation in 70% of the latently infected cats) or stressors such as lactation (40%) and moving into a new environment (18%) (Gaskell et al. 1977; Gaskell et al. 2007; Thiry et al. 2009).

3.2.6. Clinical signs

FHV has for a long time also been called feline rhinotracheitis virus and induces typically an acute upper respiratory and ocular disease with signs including fever, depression, anorexia, sneezing, coughing, nasal discharge, and conjunctivitis with ocular discharge. These discharges are in the beginning serous but become gradually mucopurulent and can crust nares and eyelids. The severity of the symptoms varies among viral strains. Clinical signs can be particularly severe in young kittens, which can also develop primary pneumonia and a viraemic state with generalised signs and eventually death. In some cats the disease may progress to a chronic immune-mediated disease such as stromal keratitis or chronic rhinosinusitis. Symptoms can be worsened by secondary bacterial infections (Stiles 2003; Gaskell et al. 2007; Thiry et al. 2009). FHV has also been identified in lesions of an ulcerative facial and nasal dermatitis (cutaneous form) and stomatitis syndrome (Hargis et al. 1999; Holland et al. 2006). Abortion is rare and not a direct consequence of viral replication but rather due to the weakening effect of the upper respiratory tract infection in the queen (Hoover et al. 1971; Verstegen et al. 2008; Thiry et al. 2009).

3.2.7. Diagnosis

The preferred method to diagnose FHV in biological samples is PCR. It is used to detect FHV DNA in conjunctival, corneal or oropharyngeal swabs, corneal scrapings, aqueous humour, corneal sequestra, blood or biopsies. PCR is more sensitive than virus isolation (Sykes et al. 1997; Vogtlin et al. 2002) or indirect immunofluorescence. In cats with recurrent disease, that shed smaller levels of virus, it is therefore more likely to yield positive results. These should however be interpreted with caution since viral DNA may also be found in asymptomatic cats (Stiles et al. 1997; Stiles 2003; Thiry et al. 2009).

Furthermore PCR can detect DNA from FHV vaccines (Maggs et al. 2005), however it is not known whether vaccine strains can be detected in vaccinated cats, nor for how long. Quantitative real-time PCR provides additional information about virus loads. High real-time PCR signals combined with positive virus isolation results are seen during acute infections and correlate well with each other. During chronic or recurrent infections, however, virus isolation is mostly negative, real-time PCR on the other hand still shows decreasing signals for a prolonged time. The analysis of consecutive samples with quantitative PCR allows the tracking of the course of infection (Vogtlin et al. 2002). PCR diagnosis is for clinicians more convenient than virus isolation or immunofluorescence, since samples do not have to be cooled during transportation and results can be obtained in less time. Serology is of very limited value in the diagnosis of FHV infection since antibody seroprevalence is high due to natural infection and vaccination and it does not correlate with disease (Gaskell et al. 2007; Thiry et al. 2009)

3.2.8. Treatment

3.2.8.1. Supportive treatment

In mild cases, good nursing care with wiping away nasal discharges and applying local ointment and eye drops can best be given at home by the owner. Food should be highly palatable and may be blended and warmed up to increase the flavour. Appetite stimulants may also be used. In severe cases, hospitalization and fluid therapy is induced and if anorexia is prolonged, a feeding tube becomes necessary. Broad spectrum antibiotics should be given in all acute cases to prevent secondary bacterial infections. Mucolytic drugs may be helpful and nebulisation with saline can be used against the dehydration of the airways. Vitamins are given, although their value is not clear (Gaskell et al. 2007; Thiry et al. 2009).

Chronic corneal ulcers caused by FHV may develop into corneal sequestra which require surgical keratectomy. Most veterinary ophthalmologists place a conjunctival flap or another type of graft material at the keratectomy site for stability reasons and in order to minimize the chances of recurrence (Stiles 2003).

3.2.8.2. Antiviral therapy

Several nucleoside analogs that had been developed against human herpesvirus and other viruses have been tested in vitro against FHV. Administration of acyclovir, which is widely used in human medicine and its prodrug valacyclovir didn't achieve therapeutic plasma concentrations in cats. In contrast, repeated administration exceeded the effective dose level (Owens et al. 1996). They don't seem to have a good activity against FHV infection and were nephrotoxic, hepatotoxic, and caused severe bone marrow depression (Nasisse et al. 1997). A number of other nucleoside analogs, such as trifluridine, idoxuridine and ganciclovir however, have shown excellent efficacy in vitro and may prove to be useful clinically. They are currently suggested in the topical treatment of ocular FHV (Gaskell et al. 2007; Thiry et al. 2009).

Feline interferon omega (feIFNω) and recombinant human interferon (rhuIFN) have proven to be effective in invitro studies against FHV (Gaskell et al. 2007; Thiry et al. 2009). FeIFNω could reduce the plaque number and plaque size in cell culture (Siebeck et al. 2006). It is licensed for the use in

cats and can be administrated subcutaneously, orally or topically as eye drops. Although there are no controlled in vivo studies on the use of interferon in FHV infections available so far, clinicians start to use it to treat FHV keratitis (Gaskell et al. 2007; Thiry et al. 2009).

L-lysine is an antagonist of arginine which has been shown to be essential for human herpes simplex virus replication. Treatment with L-lysine decreases viral proteosynthesis and has shown an inhibitory effect not only against human herpesvirus but also against FHV (Gaskell et al. 2007). When supplemented orally before conjunctival FHV infection it lessened the clinical signs (Stiles et al. 2002). It may therefore be of use in an early phase of the acute disease or to reduce its severity and the amount of virus shed during reactivation (Gaskell et al. 2007).

Bovine lactoferrin has shown an inhibitory effect on FHV when applied before or during but not after viral adsorption (Beaumont et al. 2003). Lactoferrin has the ability to bind anionic molecules and to exert antiviral activity by interacting with cellular or viral components. In the case of FHV it interacts directly with the viral particle and therefore most likely prevents the attachment and penetration of FHV into susceptible cells (Hirano et al. 2000; Gaskell et al. 2007; Jenssen et al. 2009).

3.2.9. Prevention

The European Advisory Board on Cat Diseases (ABCD) recommends all cats to be vaccinated against FHV. All currently available FHV vaccines include also other antigens, for example FCV. There are both modified-live and inactivated parenteral vaccines available. In Europe subunit vaccines and modified-live intranasal vaccines are no longer marketed. All types of vaccines induce reasonable protection against the disease, however none protect against the infection or prevent cats from becoming latently infected carriers although they can reduce viral shedding and latency load. As maternal antibodies can interfere with the development of a sufficient protection, the first immunization is usually provided at around 8-9 weeks of age with a second vaccination 2-4 week later, at around 12 weeks of age. Adult cats of uncertain vaccination status should also receive two vaccinations 2-4 weeks apart. Boosters should then be given at annual intervals for cats in high risk environments. For indoor-cats without contact to other cats, a 3-yearly interval is sufficient. Cats that have recovered from clinical signs of viral rhinotracheitis should also be vaccinated since the cause of infection will most probably not have been identified and the cat is not protected against infections with other respiratory tract pathogens (Gaskell et al. 2007; Thiry et al. 2009).

FHV is particularly a problem in cat shelters and breeding catteries. Management measures to limit the infection are there as important as vaccination. New cats should be quarantined for 2 weeks, vaccinated as soon as possible, and cross-contamination should be avoided. In queens booster vaccination before mating elevates the antibody titre and ensures a protection of the kittens for the first weeks of life (Thiry et al. 2009).

3.2.10. Disinfection

FHV is susceptible to most commercially available disinfectants, antiseptics and detergents (Eleraky et al. 2002; Thiry et al. 2009).

3.2.11. Cell culture

Alphaherpesviruses are characterized by a relative short reproductive cycle and a rapid spread in cell culture (Roizmann et al. 1992). FHV induces a characteristic cytopathic effect associated with eosinophilic intranuclear inclusion bodies (Hoover et al. 1971) and formation of multinucleated giant cells (Mochizuki et al. 1977).

3.3. Feline calicivirus

3.3.1. Taxonomy

FCV belongs to the genus Vesivirus of the family Caliciviridae. This family includes a number of animal viruses such as vesicular exanthema of swine virus, San Miguel sea lion virus and canine calicivirus (also genus Vesivirus), rabbit haemorrhagic disease virus and European brown hare syndrome virus (genus Lagovirus) (Green et al. 2000), as well as the human Norwalk virus (genus Noroviruses) (Glass et al. 2009) and Sapporo virus (genus Sapoviruses) (Hansman et al. 2007) which are both associated with an acute viral gastroenteritis (Sosnovtsev et al. 2002; Radford et al. 2007).

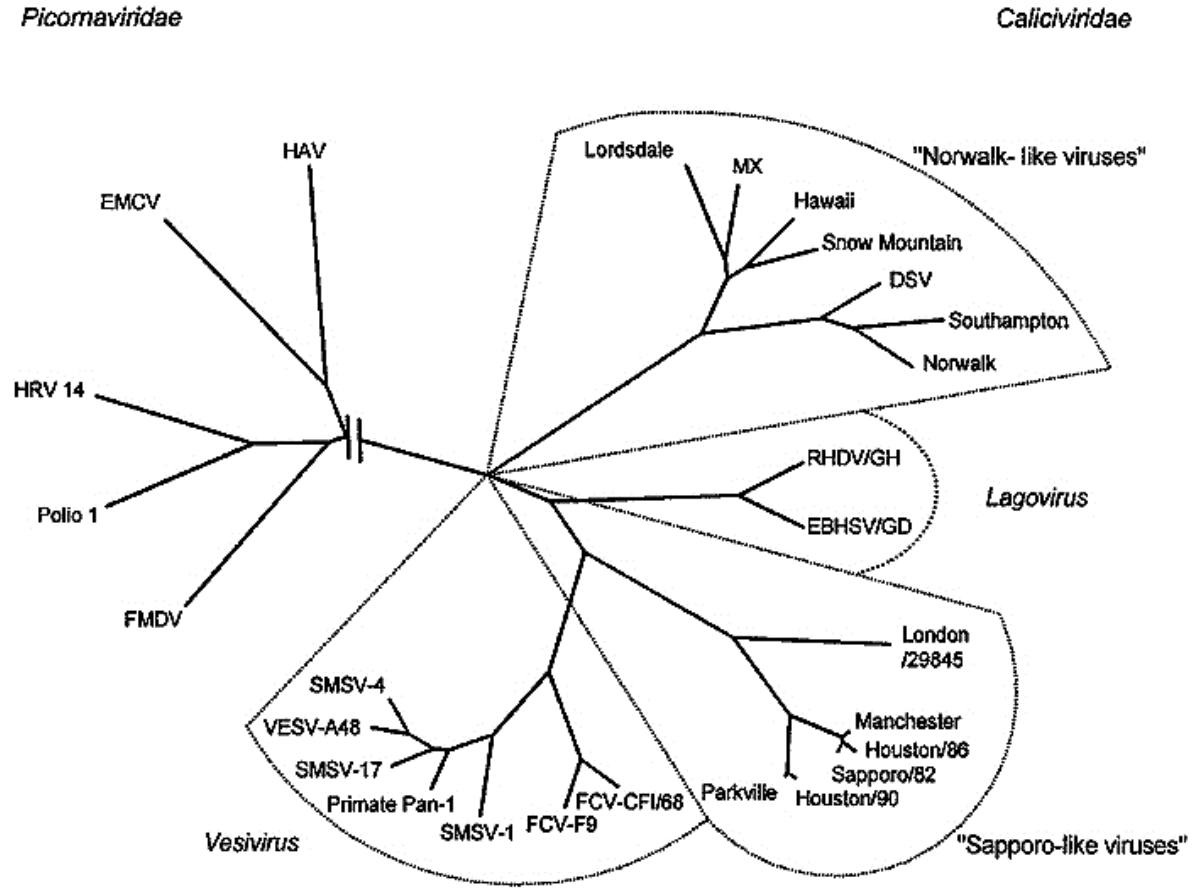

Figure 6: Phylogram of Caliciviridae (Green et al. 2000)

3.3.2. Genome

FCV has a small single-stranded, positive-sense RNA genome which is polyadenylated at the 3' end and the 5' end is bound by a small virally-encoded protein essential for its infectivity (Green et al. 2000). The genome encodes three open reading frames (ORFs). ORF 1 codes for the non-structural proteins including a viral protease and the RNA-dependent RNA polymerase. This polyprotein is posttranslationally cleaved by the viral protease. ORF 2 codes for the major capsid protein which has been divided into six regions A-F based on sequence conservation. Region A is cleaved and produces

the mature major capsid. Regions B, D and F are relatively conserved among FCV isolates, while the C and E are variable. Region E contains the major epitopes for B-cell recognition. ORF 3 encodes for a minor structural protein (Radford et al. 2007). In infected cells a subgenomic RNA could be detected which produces a precursor of the capsid protein (Zhou et al. 1994).

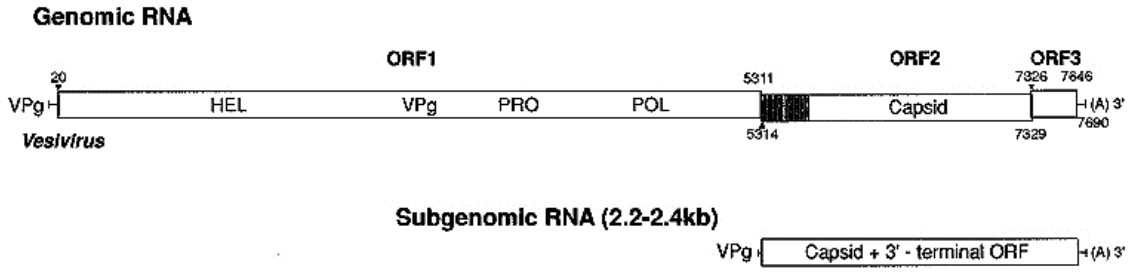

Figure 7:Calicivirus genome organisations (Green et al. 2000)

The error-prone replication by the RNA-dependent RNA polymerases lacking proof-reading, provides the genome with a high elasticity, allowing the virus to evolve quickly (Radford et al. 2007; Radford et al. 2009).

3.3.3. Structure

The caliciviruses are small nonenveloped spherical viruses. The virion is constructed from a single major capsid protein and appears to be covered with cup-shaped depressions, from which the virus received its name (calyx - a cup). No lipids or carbohydrades have been detected. (Zhou et al. 1994).

3.3.4. Epidemiology

FCV infection is widespread in cat populations. The prevalence is roughly proportional to the number of cats in a household, with the highest prevalence in large groups. It ranges from generally around 10% in small groups up to 25-40% in colonies and shelters with a high variability between individual colonies (Wardley et al. 1974; Radford et al. 2007; Radford et al. 2009). The development of an asymptomatic carrier state after recovery from the acute clinical disease is of critical importance in epidemiology. The duration of virus shedding in individual carriers is variable, ranging from months to years. Most cats have shown to be shedding after 30 days post infection, after 75 days only about 50% were still persistently infected and only a minority of cats became long-term carriers (Coyne et al. 2006).

FCV can persist in the environment for several days to weeks on dried surfaces at room temperature and even longer in colder weather conditions. Indirect transmission can therefore occur, especially in closed environments such as catteries (Radford et al. 2007).

3.3.5. Pathogenesis

Cats are infected via the nasal, oral or conjunctival routes. The oropharynx is the primary site of replication and a transient viraemia occurs 3-4 days after infection (Radford et al. 2009).

3.3.5.1. FCV-associated oral and upper respiratory tract disease

The virus induces necrosis of epithelial cells. Vesicles, typically located on the margin of the tongue, rupture and develop into ulcers with infiltration of neutrophils. Healing takes place over a period of two to three weeks. Pulmonary lesions are rare, starting initially with a focal alveolitis leading in some areas towards an acute exudative pneumonia, which can develop into a proliferative, interstitial pneumonia (Radford et al. 2007; Radford et al. 2009).

3.3.5.2. FCV-associated lameness

Lesions in joints consist of an acute synovitis with thickening of the synovial membrane and increased synovial fluid. Viral antigen has been identified in macrophage-like cells in the synovial membrane of affected joints (Levy et al. 1992; Radford et al. 2007).

3.3.5.3. FCV-associated virulent systemic disease

First reported in 1998 in California (Pedersen et al. 2000), a FCV-associated virulent systemic disease (VSD) has emerged in the United States of America and there have also been recent outbreaks in the United Kingdom and in France (Rong et al. 2006; Radford et al. 2007; Radford et al. 2009). FCV VSD is associated with a high (up to 50%) mortality rate and atypical severe clinical signs. The pathogenesis of this highly virulent infection is so far unknown (Radford et al. 2009).

3.3.6. Clinical signs

3.3.6.1. Acute oral and upper respiratory tract disease

Large numbers of different strains exist which cause a range of clinical signs. Most commonly FCV causes an acute oral and upper respiratory tract disease, which is mainly seen in kittens including the symptoms oral ulceration, ocular and nasal discharge with sneezing. Anorexia is sometimes accompanied by hypersalivation due to the oral lesions. Fever can also be observed. Usually the infection is mild and self-limiting and the erosions resolve after several days (Radford et al. 2007; Radford et al. 2009; Reynolds et al. 2009)

3.3.6.2. Chronic stomatitis

There is an association between FCV and the severe chronic oral disease, lymphoplasmatic gingivitis stomatitis (LPGS) complex, which is characterized by a proliferative/ ulzerative faucitis. Although the chronic disease could not be induced experimentally, approximately 80% of the LPGS-affected cats shed FCV compared to 20% of the controls (Knowles et al. 1989; Radford et al. 2007). The exact role of FCV remains yet unclear and other factors including other pathogens as well as host factors may be of importance (Radford et al. 2007; Radford et al. 2009).

3.3.6.3. Limping syndrome

Some FCV strains cause an acute transient lameness with fever, occurring in natural infections a few days or weeks after acute oral or respiratory signs. An acute lameness can also follow vaccination (Radford et al. 2007; Radford et al. 2009).

3.3.6.4. Virulent systemic FCV disease (VSD)

The incubation time of this highly virulent and often lethal disease is in hospitals 1-5 days and extends in home environment up to 12 days. The disease is more severe in adults than in kittens and worryingly, field vaccination does not seem to be effective. Symptoms are those of a severe acute respiratory tract disease with a systemic inflammatory response syndrome, disseminated intravascular coagulation and multiorgan failure. Mortality rates go up to 67%. Characteristic signs are pyrexia, cutaneous oedema, crusted lesions, ulcera and alopecia. Some cats suffer from hepatic necrosis and pancreatitis and show jaundice, others may show severe respiratory distress from pulmonary oedema. Thromboembolism and coagulopathy caused by the disseminated intravascular coagulation manifest as petechiae, ecchymoses, epistaxis or bloody faeces (Radford et al. 2007; Radford et al. 2009).

3.3.7. Diagnosis

Any FCV-positive result should be interpreted with caution because of the asymptomatic carrier state. VSD is diagnosed on the basis of clinical signs combined with high contagiousness, high mortality and the isolation of the same calicivirus strain from the blood of several sick cats.

3.3.7.1. Virus and antigen detection

Viral RNA can be detected by reverse-transcriptase PCR (RT-PCR) from oral swabs, blood, skin scrapings and lung tissue. PCR has the advantage that it can identify unique virus strains and this method is therefore useful in molecular epidemiology and outbreak investigation. However, there are no markers of virulence available yet (Radford et al. 2009).

Virus isolation is less sensitive however it gives proof of the presence of replicating virus. It can be performed from nasal, conjunctival and oropharyngeal swabs. Successful isolation depends on the number of infectious virions in the sample. Therefore, incorrect handling of the sample can cause inactivation and the presence of neutralizing antibodies within a sample can lead to failure of the assay. Success can be maximised if swabs are collected from both conjunctiva and oropharynx (Radford et al. 2009).

3.3.7.2. Antibody detection

FCV antibodies can be detected by virus neutralization or ELISA. In diagnostics serology is not useful because of the generally high seroprevalence due to natural infections and vaccination. It can however predict whether or not a cat is protected (Radford et al. 2009).

3.3.8. Treatment

Severely affected cats need good nursing care including cleaning away nasal discharges several times a day and applying ointment locally. Since eating may be painful, food intake should be ensured by offering strongly flavoured aromatic foods or administrating liquidized or specialized proprietary foods. In some severe cases, fluid therapy is required and where anorexia is prolonged an oesophagostomy or gastrotomy may be indicated. Non-steroidal anti-inflammatory drugs can be used to treat fever and oral pain. Broad-spectrum antibiotics with good penetration into the respiratory tract and oral mucosa are used in cats with severe disease and suspected secondary bacterial infection in

order to minimize complications. If there is a mucous nasal discharge, mucolytical drugs may be helpful and nebulisation with saline can be used against dehydration of the airways. Recombinant feline interferon omega (rFeINFω) could be shown to be effective against FCV in vitro (Ohe et al. 2008). Interferon omega (Virbagen® Omega) is licenced in Europe since 2002 for cats and is used by some clinicians in the treatment against FCV, although there are no controlled field studies so far (Radford et al. 2007; Radford et al. 2009). Most antiviral therapies used in veterinary medicine only inhibit the replication of DNA viruses or retroviruses. Ribavirin, one of the few antivirals able to inhibit FCV, seems to be toxic in cats at therapeutic levels (Radford et al. 2009).

Recommendations to treat chronic stomatitis include antibiotics combined with rigorous dental cleaning, corticosteroids and/or other immunosuppressant or immunomedulatory drugs and even total teeth extractions (Radford et al. 2009). Zeltner et al. could show reduced pain and decreased inflammation as well as an improvement of wellbeing and food uptake in cats treated subgingival with interferon omega and suggest this treatment in addition to the other measures (Zetner et al. 2004).

3.3.9. Prevention

Vaccination is the main action of prophylaxis in the cat population against FCV and is recommended by the European Advisory Board on Cat Diseases (ABCD) for all healthy cats. There are several types of vaccines such as live-attenuated and inactivated ones, monovalent or polyvalent available for parenteral use against FCV. They are considered to be safe and effective at reducing or preventing the classical FCV-associated oral/ respiratory disease, but do not protect against infection, shedding or the development of the carrier state and they do not prevent FCV VSD nor are they able to neutralize all FCV field strains (Radford et al. 2007; Radford et al. 2009). Vaccine breakdown strains (VBS), which establish infection in vaccinated cats, are problematic (Ohe et al. 2008). In the USA, but not any more in Europe, live-attenuated vaccines for intranasal use are licensed and induce local mucosal immunity, which is probably more effective against natural challenge than immunity induced by parenteral vaccines and are particularly useful when a rapid onset of protection is needed (Radford et al. 2007; Radford et al. 2009).

Usually cats are vaccinated for the first time at around 9 weeks of age and a second time 2-4 weeks later but not before 12 weeks of age. In high risk environments a third vaccination at the age of 16 weeks can be considered to ensure that maternally derived antibodies do not interfere with the development of immunity. The ABCD also recommends giving two injections 2-4 weeks apart to adult cats with unknown vaccination status. Cats living in low-risk situations, such as indoor-cats with little or no contact to other cats, can receive booster vaccinations at a 3-yearly interval however cats in high-risk environment should be revaccinated annually (Radford et al. 2009).

3.3.10. Disinfection

FCV disease is often a shelter problem. Since FCV can persist in the environment for about one month, hygiene measures are of great importance in shelter management. Effective disinfectants include sodium hypochlorite, potassium peroxymonosulfate, chlorine dioxine and commercial products

approved for calicivirus inactivation (Radford et al. 2009), quarternary ammonium compounds on the other hand are not effective (Eleraky et al. 2002).

3.3.11. Cell culture

In contrast to other members of the Caliciviridae, such as human noro- and sapoviruses, which do not grow in cell culture, FCV grows well and can therefore be studied intensively and function as a model of calicivirus molecular biology. In cell culture infected cells show a characteristic cytopathic effect (CPE) appearing as cell rounding and membrane blebbing. Like the related picornaviruses, FCV infection leads to an inhibition of cellular cap-dependent protein synthesis (shut off), achieved through the cleavage of host translation initiation factors. This mechanism may allow the virus to induce the cell's translation machinery to focus on its viral protein genome-linked (VPg-bound) RNA instead of on the cellular cap dependent mRNA translation (Willcocks et al. 2004; Radford et al. 2007).

4. Material and Methods

4.1. Cell culture

Crandell-Reese feline kidney (CRFK) cells (ATCC no. CCL-94, ATCC, Manassas, VA, USA) were maintained in RPMI 1640 (Gibco, Invitrogen, Basel, Switzerland) supplemented with 10% heat-inactivated FCS (BioConcept, Allschwil, Switzerland), 1% L-Glutamine (200mM, Gibco, Invitrogen, Basel, Switzerland), 1% penicillin/streptomycin (10'000 U/ml penicillin, 10'000 µg/ml streptomycin, Gibco, Invitrogen, Basel, Switzerland) and 0.025mM β-mercaptoethanol (Merck, Zug, Switzerland).

Felis catus whole fetus (Fcwf-4) cells (ATCC no. CRL-2787, ATCC, Manassas, VA, USA) were maintained in EMEM (ATCC) supplemented with 10% heat-inactivated FCS (BioConcept), and 1% penicillin/streptomycin (Gibco).

Peripheral blood mononuclear (PBMCs) cells (isolated from EDTA-blood of SPF cats, described in detail in section 4.2) were maintained in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FCS (BioConcept), 1% L-Glutamine (Gibco), 2.7% Sodium Bicarbonate (7.5%, Sigma-Aldrich, Steinheim, Germany), 1% penicillin/streptomycin (Gibco) and 0.025mM β-mercaptoethanol (Merck).

4.2. PBMC isolation

6-18 ml EDTA-blood were collected from SPF blood donator cats (

Table 2) and peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation. To this aim, the EDTA-blood was first centrifuged, the plasma was removed and replaced by the same volume of HBSS (Gibco). The blood cell suspension was then centrifuged on a Ficoll-Hypaque (Histopaque®-1077, Sigma-Aldrich, Steinheim, Germany) density gradient separating PBMCs from erythrocytes. Isolated PBMCs were washed once with HBSS and maintained in medium as described above (section 4.1).

Table 2: Age groups of SPF blood donator cats

Age group	Cats	Age at blood collection
Group 1	4 males (P1, S2, Q2, R3)	3 months
Group 2	4 neutered males (U2, T2, R4, Y2)	1.5 years
Group 3	3 neutered males (M3, A2, L4) 1 neutered female (J6)	4.3 years 3.8 years
Group 4	2 neutered females (2, 7)	15 years

4.3. RNA extraction

4.3.1. Automatic mRNA extraction with the Magna Pure LC (Roche)

For cytokine gene expression measurements, mRNA extractions were performed by the Magna Pure LC (Roche, Mannheim, Germany) using the mRNA isolation kit I (Roche) according to the manufacturer's instructions for blood cells with external lysis. The product was eluted in 50µl Elution Buffer.

4.3.2. Manual RNA extraction with RNeasy®Plus Mini Kit (Qiagen)

For real-time PCR assay optimization experiments, total RNA was extracted manually from pellets of $2.5x10^6$ PBMCs using the RNeasy®Plus Mini Kit (Qiagen, Hilden, Germany). Thereby, cell lysis was carried out using the QIAshredder™ (Qiagen) and genomic DNA (gDNA) was removed with the gDNA Eliminator spin column (Qiagen) according to the manufacturer's instructions. Purified RNA was eluted in 50µl Rnase-free water (provided in the kit) and stored at -20°C until further use.

4.4. Synthesis of complementary DNA (cDNA)

cDNA was produced from RNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). 10µl RNA were transcribed in a total volume of 25µl per reaction containing 6.5µl H_2O, 2.5µl cDNA synthesis buffer (10x), 2.5µl random primers (10x), 1µl 100mM dNTPs (25x), 1.25µl RNase Inhibitor (20U/µl), and 1.25µl multiscribe RT (50U/µl). Thermal conditions consisted of 10min at 25°C, 2h at 37°C, 10min at 70°C followed by cooling at 4°C until further use or storage at -20°C.

4.5. Sample production for real-time PCR assay optimization

For the production of cDNA for TaqMan® real-time PCR assay optimization, isolated PBMCs (chapter 4.2) were either treated with a combination of ConcavalinA (Sigma-Aldrich), 10µg/ml cell culture) directly after isolation and IL-2 (generous gift from Novartis, Basle, Switzerland; 50U/ml cell culture) 24h and 96h later, or were stimulated with LPS (Sigma-Aldrich, 10mg/ml cell culture) directly after isolation or left unstimulated. Treated cells were harvested 120 hours after isolation, unstimulated PBMCs directly after isolation. The cells were counted by the Sysmex XT 2000i (Sysmex, Norderstedt, Germany) and divided into pellets of $2.5x10^6$ cells, from which total RNA was extracted manually using the RNeasy®Plus Mini Kit (Qiagen) as described above in section 4.3.2. cDNA-synthesis was then performed according to the description in section 4.4.

4.6. Real-time PCR

For the detection of specific cytokine gene expression in mRNA samples and their normalization, the following TaqMan® real-time PCR assays were already available and used in the published concentrations (Table 3):

Table 3: Already available TaqMan® real-time PCR assays

Gene	Oligo	Sequences	Opt. final conc. (nM)	Reference
ABL	Forward	TGTGGCGAGTGGTGATAATACAC	300	(Kessler et al. 2009)
	Probe	CAGCATCACTAAAGGTGAAAAGCTACGAGTCCTT[1] TCCACTCACCATTCTGGTTGTAA	50	
	Reverse		900	
ACTB	Forward	CAACCGTGAGAAGATGACTCAGA	900	(Kessler et al. 2009)
	Probe	TCTCTGTACGCTTCTGGCCGCACC[2]	50	
	Reverse	CCCAGAGTCCATGACAATACCA	900	
B2M	Forward	CGCGTTTTGTGGTCTTGGT	300	(Kessler et al. 2009)
	Probe	CGGACTGCTCTATCTGTCCCACCTGGA[1]	100	
	Reverse	AAACCTGAACCTTTGGAGAATGC	900	
GAPDH	Forward	GCCATCAATGACCCCTTCAT	400	(Kessler et al. 2009)
	Probe	CTCAACTACATGGTCTACATGTTCCAGTATGATTC CA[3]	300	
	Reverse	GCCGTGGAATTTGCCGT	400	
GUSB	Forward	CTACTACGATGACATCACCATCAG	900	(Kessler et al. 2009)
	Probe	ACCAGCGTGAACCAAGACACTGGGC[2]	100	
	Reverse	CGCCTTCAACAAAAATCTGGTAA	300	
HPRT	Forward	AACTGGAAAGAATGTCTTGATTGTTG	900	(Kessler et al. 2009)
	Probe	CACTGGCAAAACAATGCAAACCTTGCTTT[2]	50	
	Reverse	GACCATCTTTGGATTATACTGCTTGA	900	
IFNγ	Forward	TGGTGGGTCGCTTTTCGTAG	900	(Leutenegger et al. 1999)
	Probe	CATTTTGAAGAACTGGAAAGAGGAGAGTGATAAA ACAAT[3]	250	
	Reverse	GAAGGAGACAATTTGGCTTTGAA	900	
IL-4	Forward	GCATGGAGCTGACCGTCAT	900	(Leutenegger et al. 1999)
	Probe	TGGCAGCCCCTAAGAACACAAGTGACAA[3]	250	
	Reverse	CGGTTGTGGCTCTGCAGA	900	
Il-6	Forward	CTCCACAAGCGCCTTC		(Taglinger et al. 2008)
	Probe	CCCTGGGAGGAGATGCCACCTCAA[1]		
	Reverse	TGCAGAGGTGAGTGGTAGTC		
IL-10	Forward	TGCACAGCATATTGTTGACCAG	300	(Leutenegger et al. 1999)
	Probe	ACCCAGGTAACCCTTAAAGTCCTCCAGCA[3]	250	
	Reverse	ATCTCGGACAAGGCTTGGC	900	
IL-12	Forward	TGGCTTCAGTTGCAGGTTCTT	300	(Leutenegger et al. 1999)
	Probe	CGGTTTGATGATGTCCCTGATGAAGAAGCT[3]	250	
	Reverse	TGGACGCTATTCACAAGCTCA	300	
RPS7	Forward	GTCCCAGAAGCCGCACTTT	50	(Kessler et al. 2009)
	Probe	CGCCGTGCACGACGCGA[4]	200	
	Reverse	CACAATCTCGCTCGGGAAAA	900	
TLR3	Forward	CAACAACTTAGCACGGCTATGG	400	kindly provided by Dr. Andrea Vögtlin* and Dr. Marco Franchini**
	Probe	AACGTGCAAACCCTAGTGGTCCTGTTGATT[3]	80	
	Reverse	AATGTGGAGGTGAGAAAGACCC	400	
TLR7	Forward	TGGTGGGTTAACCATACAGAGGTG	400	(Ignacio et al. 2005)
	Probe	ACTTGGCCACAGATGTGACTTGTGTG	80	
	Reverse	GAGAAAGAGCCACCGATACGGAAA	400	

TLR9	Forward	TACGATGCCTTTGTGGTCTTCGAC	400	(Ignacio et al.
	Probe	TCTTTGAGAACCTGTGGGCCTCAGTT	80	2005)
	Reverse	ACAAACAGCATCTTGCGGCT	400	
TNFα	Forward	CACATGGCCTGCAACTAATC		(Taglinger et al.
	Probe	TCTCGAACTCCGAGTGACAAGCCA[1]		2008)
	Reverse	AGCTTCGGGGTTTGCTACTAC		
YWHAZ	Forward	ACAAAGACAGCACGCTAATAATGC	900	(Kessler et al.
	Probe	CGATGTCCACAATGTCAAGTTGTCTCTCAG[2]	150	2009)
	Reverse	CTTCAGCTTCATCTCCTTGGGTAT	900	

[1] 5' FAM/3' BHQ-1; [2] 5' Yakima-Yellow/3' BQH-1; [3] 5' FAM/3' TAMRA; [4] 5' TET/3' TAMRA

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For the measurement of additional cytokine systems, new TaqMan® real-time PCR assays were established (Table 4). Primers and TaqMan™ probes were designed using Primer Express™ software (versions 2 and 3, Applied Biosystems). The sequences were retrieved from Ensemble (http://www.ensemble.org) and GeneBank (http://www.ncbi.nlm.nih.gov). Primers were synthesized commercially (Microsynth, Balgach, Switzerland).

Table 4: Developed TaqMan® real-time PCR assays

Gene	Accession Number	Oligo	Sequences
Granzyme B	EU153367	Forward	CCACCCAGACTATAATCCAAAGAA
		Probe	CCAACGACATCATGTTACTGCAGCTGG[1]
		Reverse	CAGTCAGCTTGGCCTTTTTCA
IFNα	AY117395	Forward	CACGTGACGAACCAGAAGATCTT
		Probe	ACTTCTTCTGCACAGAGGCGTCCTCG[1]
		Reverse	GAGGGTGGTGTTCCAAGCA
IFNα3	AY117393	Forward	CGTGACGAACCAGGAGATCTTC
		Probe	same as IFNα
		Reverse	same as IFNα
IFNα7	AB094996	Forward	CACGTGACCAACCAGAAGATCTT
		Probe	same as IFNα
		Reverse	same as IFNα
IFNα14	AB095003	Forward	CGTCTGCTCTCTGGGTTGTG
		Probe	CCTGCCTCAGACCCACGGCC[1]
		Reverse	ATTTGTCCCAGGAGCGTCAA
IFNβ	AB021707	Forward	TGGAATGAGACCACTGTTGAGAA
		Probe	CTCCTTGCGACACTCCACTGGCAG[1]
		Reverse	GGATCGTTTCCAGGTGTTCCT
IFNω	DQ420220	Forward	CGCAGGTTAGCAGGGACAAC
		Probe	CGGAGACTGTCCCCTTTCTTGTGCC[1]
		Reverse	GGGAAGCGGAAGTCTTTTCTG
Il-15	ENSFCAG00000011861	Forward	AGTGATGTTCATCCCAATTGCA
		Probe	TTCGCTTGAGTCCAAAAATGCGACCA[1]
		Reverse	ACCGCTGTTTGCTAGGATAATAATG
Mx	NM-002462	Forward	ACCAGAGCTCGGGCAAGAG
		Probe	CCTTCCCAGAGGCAGCGGTATTGTC[1]
		Reverse	TTCAGCACCAGAGGACACCTT
Perforin	EU032539	Forward	TTCGCGGCCCAGAAGAC
		Probe	TTCCACGACCAGTACAGCTTCAGCACTG[1]
		Reverse	GTGAGAGCTGTAGAAGCGACATTC
TLR8	ENSFCAG00000007243	Forward	GCTCCAGCTGTTTCCTCATC
		Probe	CCAGTTGCTCGACTTAAGTGG[1]
		Reverse	GAGGCTGTTGGTCAAAGAGG

[1] 5' FAM/3' TAMRA

All PCR mastermixes and samples were pipetted by the automatic pipettor Cas-1200 (Corbett, Mortlake, Australia). PCR reactions were performed using 5µl cDNA of the sample in a total volume of 25µl per reaction, containing 12.5µl TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) per reaction, the appropriate volumes of forward and reverse primers and probe and H_2O. All PCR reactions were performed on a Rotor-Gene 6000 real-time rotary analyser (Corbett, Mortlake, Australia). Thermocycling conditions consisted of an initial denaturation step of 20s at 95°C followed by 45 cycles of 95°C for 3s and 60°C for 30s.

4.7. Optimization and evaluation of quantitative real-time PCR assays

Primer and probe concentrations were optimized on five-fold serial dilutions (namely 5^{-1}, 5^{-2}, 5^{-3}, 5^{-4}) or in the case of IL-6 two-fold (2^0, 2^{-1}, 2^{-2}, 2^{-3}, 2^{-4}) serial dilutions of cDNA derived from PBMCs

(described in detail in section 4.5) containing 1:300 diluted salmon sperm DNA (Invitrogen). Table 5 indicates which PBMC stimulation had been used for the optimization of the different TaqMan® real-time PCR assays.

Table 5: PBMC treatment for cDNA production for TaqMan® real-time PCR assay optimization

PBMC treatment	**TaqMan® real-time PCR assays**		
ConA/ IL-2	IFNα	IFNω	Mx
	IFNα3	IL-4	Perf
	IFNα7	IL-10	TLR3
	IFNα14	IL-12	TLR7
	IFNβ	IL-15	TLR8
	IFNγ	GranzB	TLR9
LPS	IL-6		
	TNFα		
untreated	GUSB		
	YWHAZ		

Primer end concentrations of 300, 600 and 900nM or 200, 400 and 800nM were evaluated using 250nM probe end concentration. Each concentration was run in quadruplicates using 5µl sample in a total volume of 25µl per reaction on a RotorGene™ 6000 real-time rotary analyzer (Corbett, Mortlake, Australia) (for details see section 4.6). The performance of the assays was further optimized by analyzing the amplification of the samples using the optimal primer concentration combined with 3 different probe end concentrations, namely 50, 150, and 250nM. Calculation of the coefficients of correlation, slopes and amplification efficiencies $E=10^{(-1/slope)}-1$ using the RotorGene™ 6000-software 1.7 (Corbett, Mortlake, Australia) enabled to select optimal conditions for each assay.

4.8. Selection of the house keeping genes

In order to select the two house keeping genes best suited for gene expression normalisation, the stability of five housekeeping genes, ABL, ACTB, GUSB, RPS7, and YWHAZ (Kessler et al. 2009) was tested in two experiments and compared. In the first experiment, the sample cDNA was derived from CRFK cells of four treatment groups (CpG, R-848, Poly I:C, RPMI) infected with feline corona virus (FCoV) Wellcome strain. 10^4 CRFK cells had been seeded per well into a 96-well plate in triplicates for each treatment group and incubated for 24 hours at 37°C, 5% CO_2. The cells were then treated with three different immune response modifiers namely CpG, R-848 or Poly IC (Alexis biochemicals, Enzo Life Sciences AG, Lausen, Switzerland) (concentrations are shown in Table 6) or with medium alone and again incubated for 24h under the same conditions. After pre-treatment, the cells were infected with 5x $TCID_{50}$ FCoV (1:30 dilution of stock virus) previously titrated with the method described below on the CrFK cells (section 4.11.3), and incubated for 72h at 37°C, 5% CO_2. The cells were harvested and lysed in mRNA-lysis buffer (Roche) and stored at -20°C until mRNA extraction with the Magna Pure LC (Roche) (section 4.3.1) and cDNA synthesis (section 4.4). In the second experiment, the housekeeping genes were tested in cDNA derived from PBMCs treated with

CpG VR-1 or medium alone. The two most stable housekeeping genes among the treatment conditions were selected in each experiment using BestKeeper software tool version 1, an Excel-based tool using pair-wise correlation analysis (Pfaffl et al. 2004), and GeNorm, a visual basic application applet for Microsoft Excel version 3.5 (Vandesompele et al. 2002).

Table 6: Concentrations of Immune Response Modifiers

IRM	Published conc. (µg/ml)	Recommended conc. (µg/ml)	Diluent	Initial conc. (µg/ml)	Final conc. (µg/ml), (1x)
CpG VR-1	3*	1-2.5**	PBS[1]	66.44	2.5
CpG VR-2			PBS[1]	66.44	2.5
R-848	1*		DMSO	1	5
Poly I:C	50*	10-25**	PBS[1]	1	25

* (Hansmann et al. 2008); ** Alexis Biochemicals; [1] endotoxin-free

4.9. Measurement of cytokine expression in stimulated PBMCs by quantitative real-time PCR

PBMCs were lysed in mRNA lysis buffer (Qiagen) after stimulation (section 4.10), mRNA was extracted with the Magna Pure LC (Roche) (section 4.3.1) and cDNA was synthesized (section 4.4). The cDNA was diluted 1:3 in H_2O already containing salmon sperm DNA (Invitrogen) diluted 1:300. PCR measurements were performed in duplicates according to the protocol described in section 4.6. The results were normalized to the two best housekeeping genes selected as reference genes by the experiments outlined in section 4.8. Gene expression factors between treated and non-treated samples (negative controls) were calculated using GeNorm.

4.10. Production of potentially antiviral cell culture supernatant

4.10.1. CpG VR-1, CpG VR-2

CpG VR-1 (Alexis Biochemicals) (ggGGGACGATCGTCgggggG) is a type A CpG-ODN, known to induce high amounts of type I IFN in PBMCs (Krug et al. 2001). It has a mixed backbone containing phosphorothiate modifications at both ends and phosphodiester linkages in the center portion, a poly G tail at both ends and central CG motifs within flanking palindromic sequences (Guzylack-Piriou et al. 2004). CpG VR-2 (ggGGGAGCATGCTCgggggG) is the GC control to CpG ODN 2216 (Krug et al. 2001).

4.10.2. Evaluation of the optimal CpG concentration

For experiments evaluating the optimal CpG concentration for maximal stimulation of the innate immunity, $1.2x10^6$ PMBCs, isolated from one SPF blood donor cat (Y2: neutered male, 15 months old), were cultured per well in 12-well cell culture plates either in 1.4ml complete RPMI medium

containing CpG VR-1 (Alexis Biochemicals) in 1x, 2x, 4x and 8x the concentrations shown in Table 6, or as a control in 1.4ml complete RPMI medium. After a 24 hour incubation at 37°C, 5% CO_2, the cultures were harvested and centrifuged for 10min at 140 x g. The supernatants were aliquoted in separate tubes and stored at -20°C. The cells were divided into 2 equal aliquots, each lysed with 300µl mRNA lysis buffer (Qiagen) and stored at -20°C until mRNA extraction. The expression factor of three genes of interest, namely IFNα, IFNω and TNFα were compared in order to find the maximally stimulating concentration.

4.10.3. Evaluation of the optimal CpG incubation-time

In order to evaluate the optimal harvesting time point, PBMCs (cat Y2: neutered male, 15 months old) were treated directly after isolation with CpG VR-1 in a final concentration of 2x (Table 6) for 24h, followed by incubation with ConA for 8h and IL-2 stimulation, which was repeated after 3 days to ensure PBMC survival in vitro. Cells and supernatants were harvested with the same method as described above (section 4.10.2) after incubation times of 24h, 32h, 56h, 104h, and 128h.

4.10.4. Main experiments: CpG VR-1 stimulation of PBMCs

Due to its optimal stimulation ratio, PBMC stimulation was carried out with CpG VR-1 treatment in the final concentration of 2x for 24h with the same procedure as described in section 4.10.2 in all further experiments. For the supernatant production for viral inhibition experiments on Fcwf-4 cells, an additional control was included by treating PBMCs with the non-stimulating CpG VR-2.

4.11. Viruses

4.11.1. Virus strains

The following viruses were used for viral inhibition assays: Feline calicivirus (FCV) F9 strain, feline herpesvirus (FHV) ZH5-04 strain, and vesicular stomatitis virus (VSV) Indiana strain as a reference. FCV and FHV were kindly provided by Veterinaria, Zurich, Switzerland. VSV Indiana strain was kindly provided by PD Dr. Monika Engels of the Institute of Virology at the Vetsuisse Faculty of the University of Zurich.

4.11.2. Preparation of virus stock

FCV and FHV were grown once on CRFK cell cultures in 850 cm^2 rollerbottles for 48 hours. Cell supernatants were collected, centrifuged for 10 min at 140 x g and passed through a filter with 200nm pore size (Filtropur V25 0.2, Sarstedt, Inc. USA) under vacuum. Aliquots were stored at -80°C. FHV was homogenized by immersion for one minute into an ultrasonic bath.

VSV was grown on Vero cell cultures in two 75 cm^2 cell culture flasks for 48 hours. Cell supernatant was collected, centrifuged for 10 min at 140 x g and passed through a 0.2 µm filter. Aliquots were stored at -80°C.

4.11.3. Virus titration

FHV and FCV were titrated on both CRFK and Fcwf-4 cells in 96-well plates in order to evaluate the optimal concentration for infection in later experiments. $2x10^4$ CRFK cells or $7x10^4$ Fcwf-4 cells, respectively, had been seeded in 100µl of complete medium per well in separate 96-well cell culture plates and grown for 24 hours at 37°C, 5% CO_2. The medium was then discarded and cells were inoculated with five-fold serial dilutions of virus stock solution in a total of 100µl complete medium starting with undiluted virus. Each virus inoculation was performed in quadruplicates. Cell cultures were incubated for 24 hours at 37°C, 5% CO_2 and the highest dilution inducing approximately 100% cytopathic effect (CPE) was evaluated using the Plaque Assay (Vogel et al. 2001), see section 4.12.2. The wells treated with undiluted virus were used as virus controls and the means of their OD values defined as 100% CPE. In turn, the uninfected negative control wells were considered showing 0% CPE. The percentage of CPE in the remaining virus dilutions were calculated according to Equation 1. VSV and FCoV had been previously titrated with the same method on these cell lines. VSV stock virus was diluted $1:10^6$ for experiments on CrFK cells and $1:10^5$ for Fcwf-4 cells. FCoV was diluted 1:30 for experiments on CrFK cells.

Equation 1:

$$CPE = \left(1 - \frac{mean_x - mean_{VC}}{mean_{neg.C} - mean_{VC}}\right) x\,100$$

x: sample, VC: virus control, neg.C: negative Control

4.12. In vitro viral inhibition assays

4.12.1. Preparation, treatment and infection

Assays were carried out in separate 96-well plates for each virus and each cell line. 10^4 CRFK or 10^4 Fcwf-4 cells in 100µl complete medium were seeded per well. The cell cultures were incubated for 24 hours at 37°C, 5% CO_2. The medium was discarded from each well and cells were treated with 100µl of the supernatants produced as described in section 4.10. Each supernatant was tested in duplictates. Cells treated with medium alone were included as positive controls and uninfected cells as negative controls. A 10-fold serial dilution of feline rIFNα (RnD Systems, Abingdon, UK) starting with a 1:10 dilution of the stock solution (equalling 1000U/ml) was added as standard positive treatment control in each experiment. The cell cultures were treated for 24 hours at 37°C, 5% CO_2. Total supernatant was discarded from each well and cells were infected with 100µl/well of either FHV, FCV or VSV at dilutions determined by viral titration experiments (section 4.11.3). At the time point of 16 hours post infection, cytopathic effects (CPE) were evaluated microscopically in each well and the plaque assay (Vogel et al. 2001) was performed as described below.

4.12.2. Plaque assay (Vogel et al. 2001)

The supernatants were removed and each well was washed with 100µl HBSS and placed for 15 sec on an orbital shaker (Vari-Shaker, Dynatech; speed adjusted to 7 on a scale of 12 positions) in order

to detach cell debris. The washing step including the shaking was repeated thrice. The cells were fixed with 100µl of 5% formalin in each well for 40 min at room temperature and stained with 100µl crystal violet solution per well for 10 min. The plates were rinsed with tap water and allowed to dry. For reading of the samples spectrophotometrically, 100µl of 100% methanol were added to each well in order to elute the dye from the fixed cells and the absorbance was read at 595nm on a SpectraMax Plus384 (Molecular Devices, Bucher Biotec AG, Basel, Switzerland) microtiter plate reader.

4.13. Statistics

Statistical analysis was performed with GraphPad Prism for Windows, Version 3.0 (GraphPad software, San Diego, CA, USA). Normalized cytokine expression factors were tested for statistical differences between treated and untreated samples using a non-parametric Wilcoxon signed rank test for paired samples. Differences among age-groups were evaluated with the Kruskal-Wallis and the Dunn's Multiple Comparison Test. In viral inhibition experiments, differences between treatments with CpG VR-1/ CpG VR-2/ and negative supernatants were evaluated with a non-parametric Wilcoxon signed rank test for paired samples and differences between negative supernatants and medium were assessed with a Mann-Withney test for unpaired samples. Correlations between either the expression factors of two different genes or one gene and the viral inhibition (OD values) were evaluated by calculating the Spearman correlation coefficients (r). Significance was accepted if $p < 0.05$.

5. Results

5.1. TaqMan® real-time PCR assays

5.1.1. Optimization of primer and probe concentrations

Optimal primer and probe concentrations were evaluated using cDNA containing 1:300 diluted salmon sperm DNA (section 4.5). Concentrations found to yield optimal amplification are presented in Table 7.

Table 7: Optimal final concentration of primers and probe

Real-time PCR assay	Forward Primer (nM)	Reverse Primer (nM)	Probe (nM)
Granzyme B	600	600	250
IFNα	300	300	250
IFNα3	900	900	250
IFNα7	600	600	150
IFNα14	600	600	150
IFNβ	900	900	50
IFNω	600	600	50
IL-6	800	800	250
IL-15	400	400	50
Mx	900	900	250
Perforin	900	900	250
TLR8	400	400	80
TNFα	800	800	50

5.1.2. Determination of amplification efficiencies

The amplification efficiencies ($E=10^{(-1/slope)}$ -1) of all TaqMan® real-time PCR assays were calculated (Table 8).

Table 8: Amplification efficiencies of all TaqMan® real-time PCR assays

Real-time PCR assay	Correlation Coefficient	Slope	AE $E=10^{(-1/slope)}$ -1
ABL	0.99396	-3.152	1.08
ACTB	0.99142	-3.204	1.05
B2M	0.99774	-3.331	1.00
GAPDH	0.99076	-3.006	1.15
Granzyme B	0.99474	-3.400	0.97
GUSB	0.98872	-3.340	0.99
HPRT	0.97302	-3.271	1.02
IFNα	0.99514	-3.221	1.04
IFNα3	0.99567	-3.159	1.07
IFNα7	0.99154	-3.048	1.13
IFNα14	0.98905	-3.327	1.00
IFNβ	0.92435	-3.400	0.97
IFNγ	0.98013	-3.367	0.98
IFNω	0.99583	-3.161	1.07
IL-4	0.95069	-3.434	0.96
IL-6	0.93898	-3.349	0.99
IL-10	0.96395	-3.544	0.91

IL-12	0.94235	-3.372	0.98
IL-15	0.96227	-3.319	1.00
Mx	0.99780	-3.323	1.00
Perforin	0.99404	-3.379	0.98
TLR3	0.98013	-3.170	1.07
TLR7	0.98132	-3.355	0.99
TLR8	0.95657	-3.299	1.01
TLR9	0.89770	-3.524	0.92
TNFα	0.99317	-3.386	0.97
RPS7	0.98971	-3.419	0.96
YWHAZ	0.99496	-3.346	0.99

5.2. Selection of the house keeping genes

The house keeping genes providing the most stable expression were evaluated in two different experiments. In the first, CRFK cells had been pre-treated with four different IRMs and infected with FCoV, in the second experiment PBMCs of 6 cats were treated either with CpG or with medium. The house keeping genes were ranked according to their stability by BestKeeper software tool version 1 (Pfaffl et al. 2004), and GeNorm version 3.5 (Vandesompele et al. 2002) for both experiments. (Table 9)

Table 9: Stability ranking of the house keeping genes (HKGs)

Experiment	**Calculation program**	**Ranking of the HKGs (starting with the best)**
IRMs/FCoV, CRFK	BestKeeper GeNorm	GUSB>YWHAZ>RPS7>ACTB>ABL GUSB=YWHAZ>ACTB>RPS7>ABL
CpG/medium, PBMC	BestKeeper GeNorm	ACTB>GUSB>RPS7>YWHAZ>ABL ABL=YWHAZ>RPS7>ACTB>GUSB

According to these results and to further arguments discussed in section 6.3.2, GUSB and YWHAZ were selected for normalization in further experiments.

5.3. Cytokine gene expression in stimulated PBMCs

5.3.1. Evaluation of the optimal CpG concentration

In order to evaluate the optimal treatment concentration of CpG VR-1, the mRNA expression factors of three selected cytokines, namely IFNα, IFNω and TNFα, were measured in PBMCs after stimulation with four different concentrations (1x, 2x, 4x, 8x of the concentration shown in Table 6) of CpG VR-1 for 24 hours.

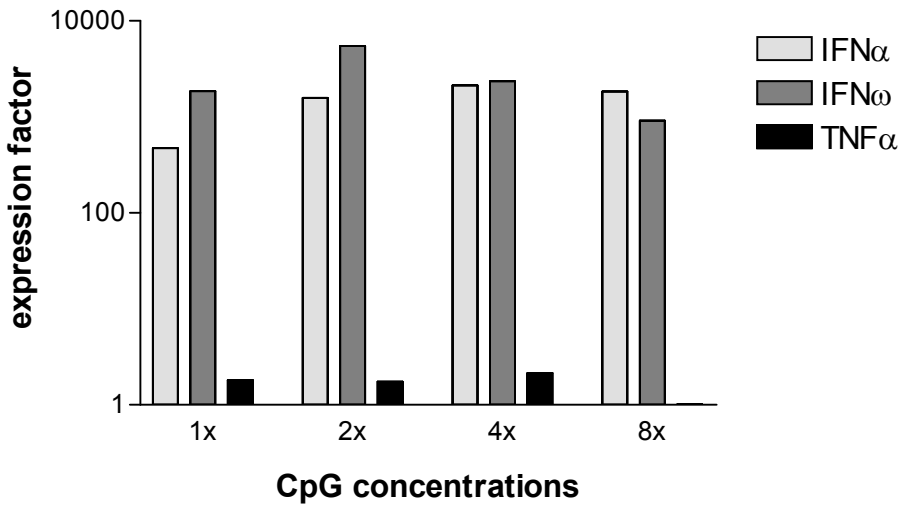


Figure 8: Cytokine expression for the evaluation of the optimal CpG concentration

Feline PBMCs of one cat were stimulated for 24 hours with four different concentrations of CpG VR-1 (1x, 2x, 4x, 8x the final concentration of 2.5μg/ml) or with medium alone as negative control. mRNA expression of three innate cytokines was measured by real time PCR and normalized to two feline housekeeping genes (GUSB and YWHAZ). Expression factors were calculated by GeNorm comparing results of samples treated with CpG VR-1 to those of the negative control.

5.3.2. Evaluation of the optimal CpG incubation-time

In order to evaluate the optimal harvesting time point for supernatants and PBMCs after CpG stimulation (concentration 2x), the mRNA expression factors of six selected cytokines, namely IFNα, IFNω, TNFα, IL-6, IFNγ and IL-12, were measured in PBMCs at different time points of incubation (24h, 32h, 56h, 104h, 128h).

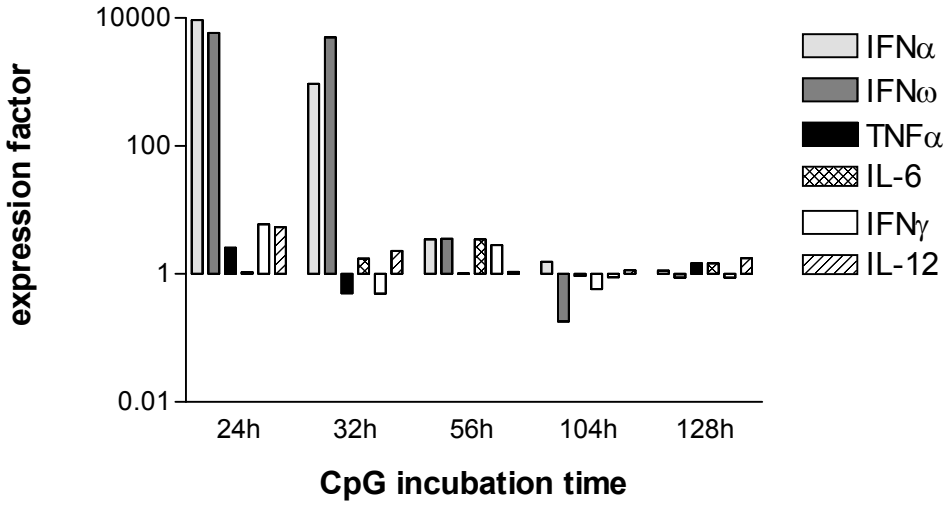


Figure 9: Cytokine expression for the evaluation of the optimal CpG incubation time

Feline PBMCs of one cat were stimulated with a concentration of 2x 2.5μg/ml CpG VR-1 or with medium alone as negative control. After different time points of incubation (24h, 32h, 56h, 104h, 128h) mRNA expression of six innate cytokines was measured by real time PCR and normalized to two feline housekeeping genes (GUSB and

YWHAZ). Expression factors were calculated by GeNorm comparing results of samples treated with CpG VR-1 to those of the negative control.

5.3.3. Main experiment: Expression of 14 genes in PBMCs of 14 cats

Cytokine mRNA expression was measured in PBMCs from 14 SPF blood donator cats of different age groups (for details on the groups see

Table 2) either treated with CpG VR-1 for 24 hours or left unstimulated. The gene expression was measured by TaqMan® real-time PCR and expression factors between treated and untreated samples were calculated by GeNorm (Vandesompele et al. 2002), which normalizes all results with two reference genes (GUSB, YWHAZ) and takes into consideration the amplification efficiency of each assay.

For statistical analysis, where normalized values for both treated and untreated samples were required, ratios were calculated between the ct-value of the gene of interest and the mean of the ct-values of the two housekeeping genes from the same sample (Equation 2).

Equation 2:

$$ratio = \frac{ct_{gene\ of\ interest}}{(ct_{GUSB} + ct_{YWHAZ})/2}$$

A non-parametric Wilcoxon signed rank test for two paired samples was performed for each gene of interest to evaluate statistical differences between treated and untreated samples.

mRNA expression of type I IFNα, -β, and -ω, as well as type I IFN-inducible gene Mx was highly significantly upregulated. IL-12, IL-15, GrzB, IL-6 and TNFα were also significantly induced (Figure 10). For the type I IFNs, the range of induction was quite broad with PBMCs from two cats of group 1 (3 months old) failing to show stimulation or even indicating a down-regulation of these genes. However, no significant difference between age groups could be shown for any of the tested genes except for IFNβ between group 1 and group 2 (Figure 11a-n).

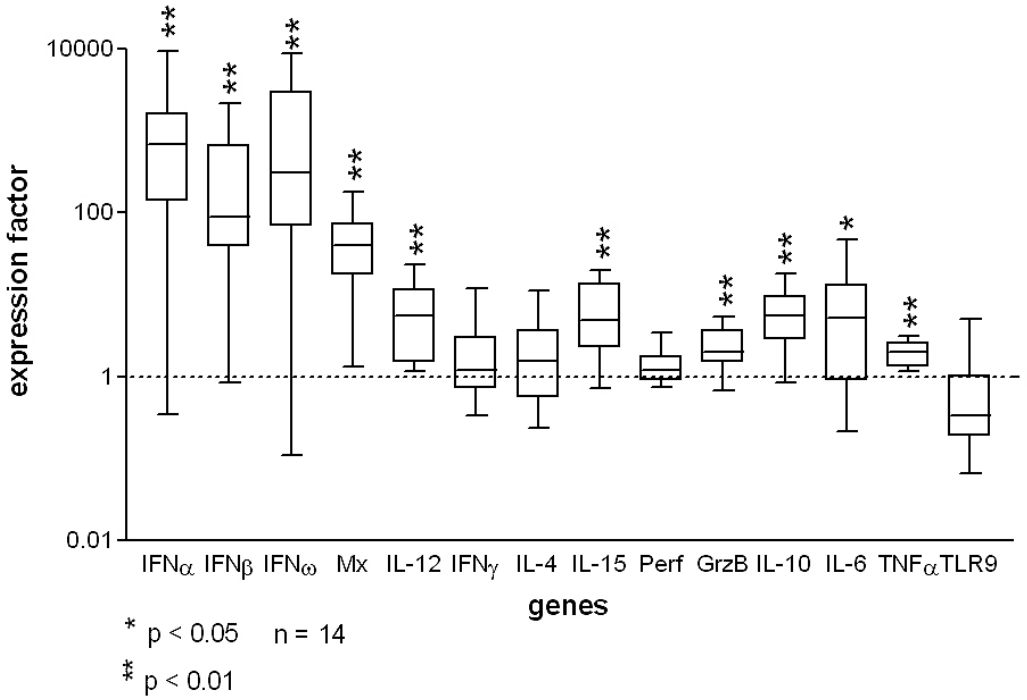


Figure 10: Cytokine expression factors

PBMCs of 14 cats were stimulated for 24h with a concentration of 2x 2.5μg/ml CpG VR-1 or with medium alone as negative control. mRNA expression of 14 genes of interest concerning innate immunity was measured by real time PCR and normalized to two feline housekeeping genes (GUSB and YWHAZ). Expression factors were calculated by GeNorm comparing results of samples treated with CpG VR-1 to those of the negative control. A non-parametric Wilcoxon signed rank test for two paired samples was performed for each gene to evaluate statistically significant differences between treated and untreated samples.

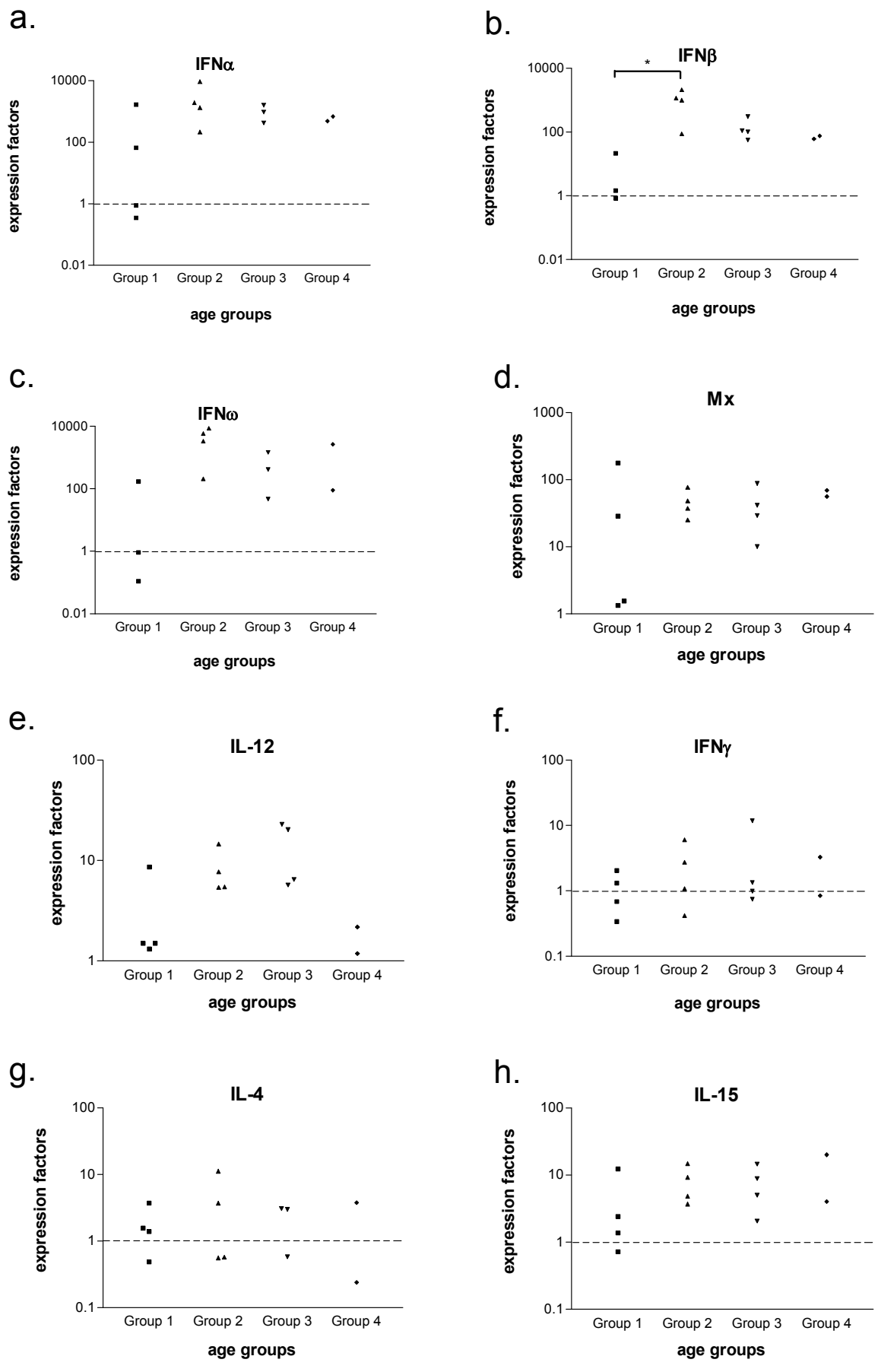


Figure 11a-n: Gene expression factors in different age groups

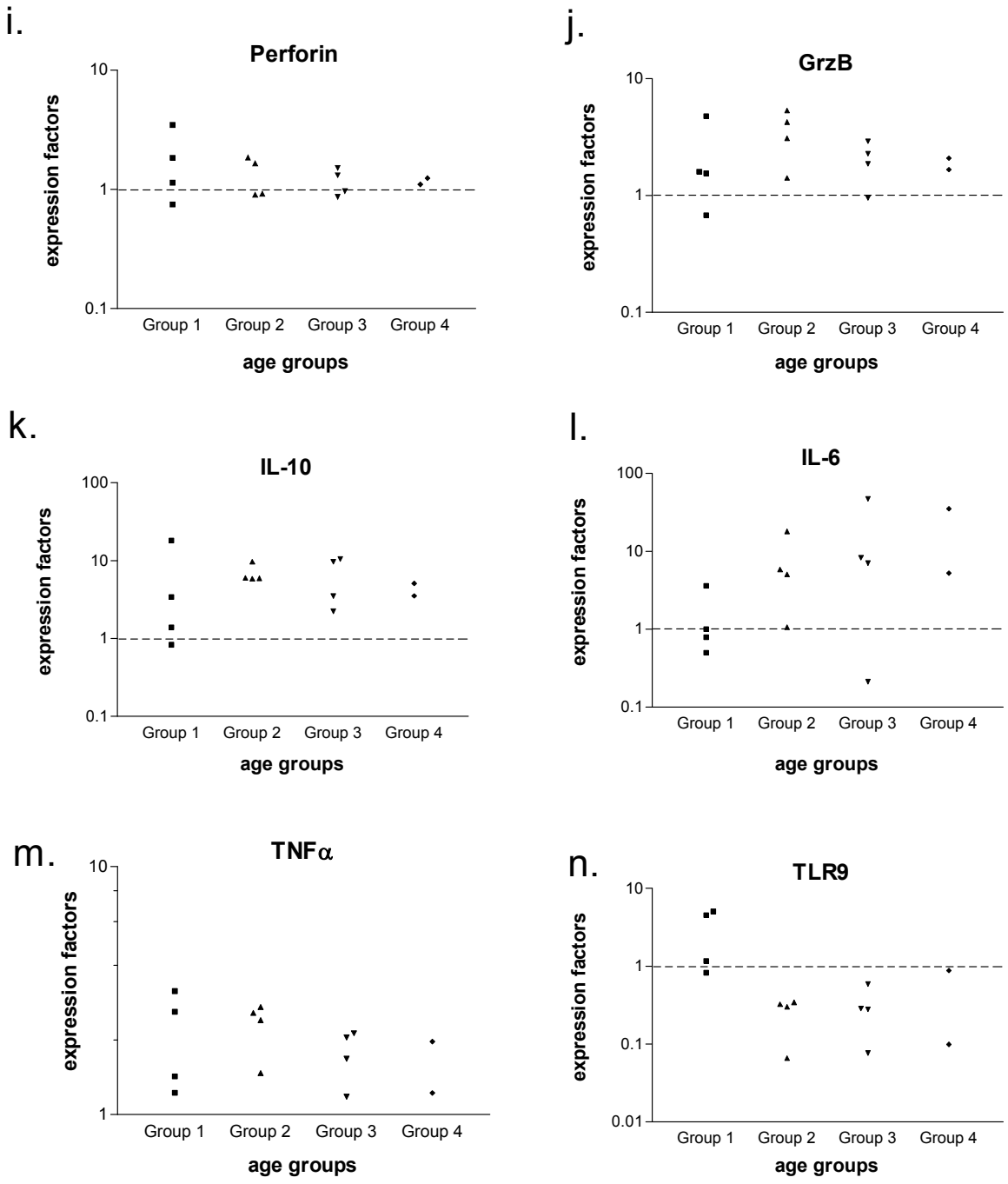


Figure 11a-n: Gene expression factors in different age groups

Gene expression factors of the 14 genes of innate immunity shown in Figure 10 displayed for each gene separately presenting the different age groups of the cats (group 1: 4 cats of 3 months; group 2: 4 cats of 1.5 years; group 3: 4 cats of 3.8-4.3 years; group 4: 2 cats of 15 years). PBMCs had been stimulated for 24h with a concentration of 2x 2.5µg/ml CpG VR-1 or with medium alone as negative control. mRNA expression of 14 genes of interest concerning innate immunity was measured by real time PCR and normalized to two feline housekeeping genes (GUSB and YWHAZ). Expression factors were calculated by GeNorm comparing results of samples treated with CpG VR-1 to those of the negative control. Differences among age-groups were evaluated with the Kruskal-Wallis and the Dunn's Multiple Comparison Test.

5.4. IFN and Mx mRNA expression in PBMCs

The correlation between the induction of type I IFNα, -β, -ω or type II IFNγ and the stimulation of the Mx gene in PBMCs were evaluated with GraphPad Prism and the Spearman correlation coefficients (r) and the p-values were calculated. A significant correlation between IFNα/Mx, IFNβ/Mx, and IFNω/Mx could be shown. There was no correlation between IFNγ and Mx induction (Figure 12).

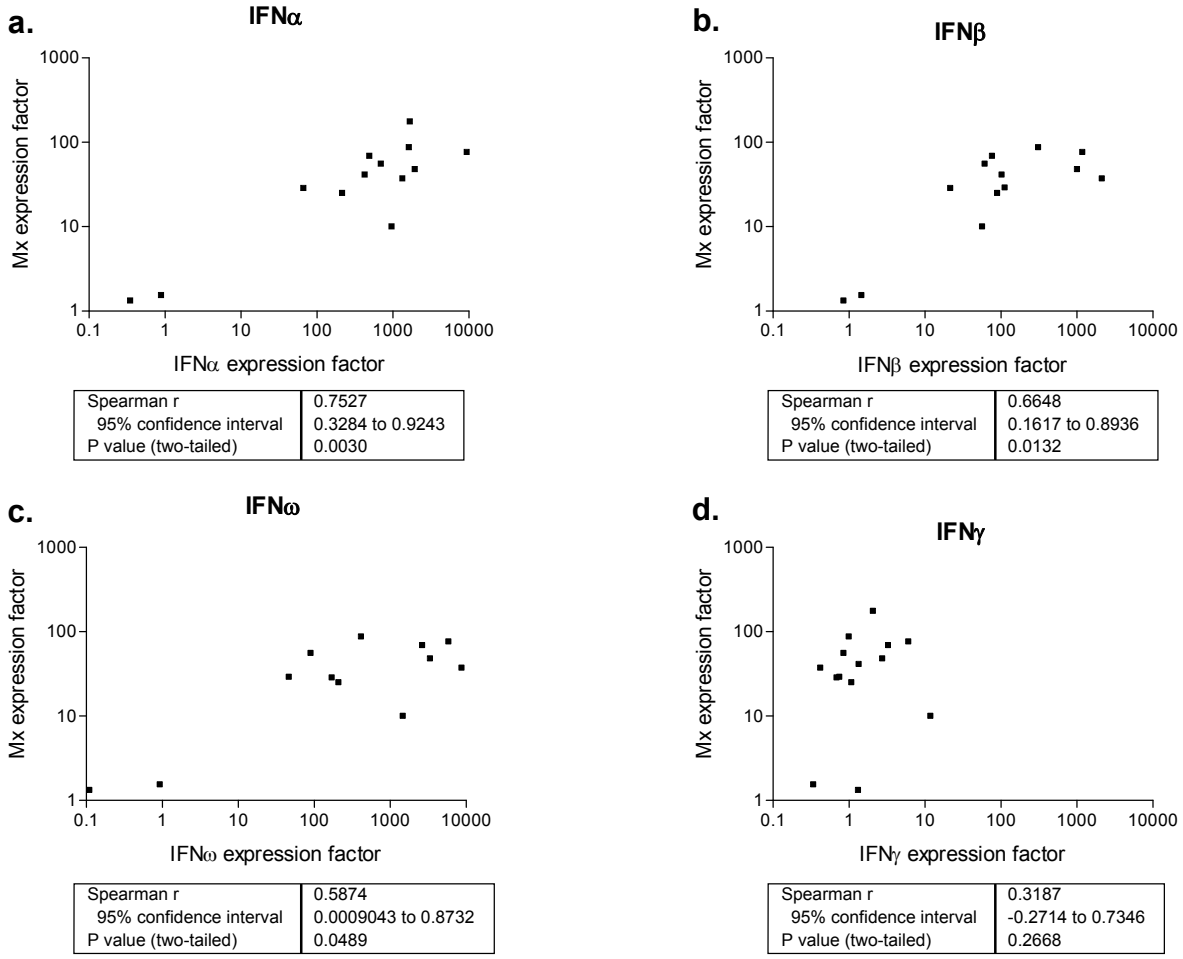


Figure 12a-d: Correlation of IFN and Mx mRNA expression in PBMCs

Gene expression factors of type I interferon (a: IFNα; b:IFNβ; c:IFNω) and type II interferon (d: IFNγ) calculated by GeNorm comparing results of samples of PBMCs treated with CpG VR-1 to those of the negative control and shown already in Figure 10 and Figure 11 were tested with GraphPad Prism Version 3.0 for correlation to expression factors of Mx gene in the same PBMCs. Each dot represents one cat.

5.5. Mx gene expression in supernatant-treated cells

Mx gene expression factors were calculated with GeNorm (Vandesompele et al. 2002) by comparison of supernatant-treated cells (CpG VR-1-, CpG VR-2- or neg. supernatant) with untreated cells which had received only medium. An upregulation of the Mx gene expression could be measured in all cells stimulated with supernatants of PBMCs previously treated with CpG VR-1 and to a lesser degree in cells treated with CpG VR-2 supernatants. The control supernatants (neg) of untreated PBMCs on the

other hand did not induce Mx gene expression in cells. This experiment was performed on CRFK cells (Figure 13) as well as on Fcwf-4 cells (Figure 14).

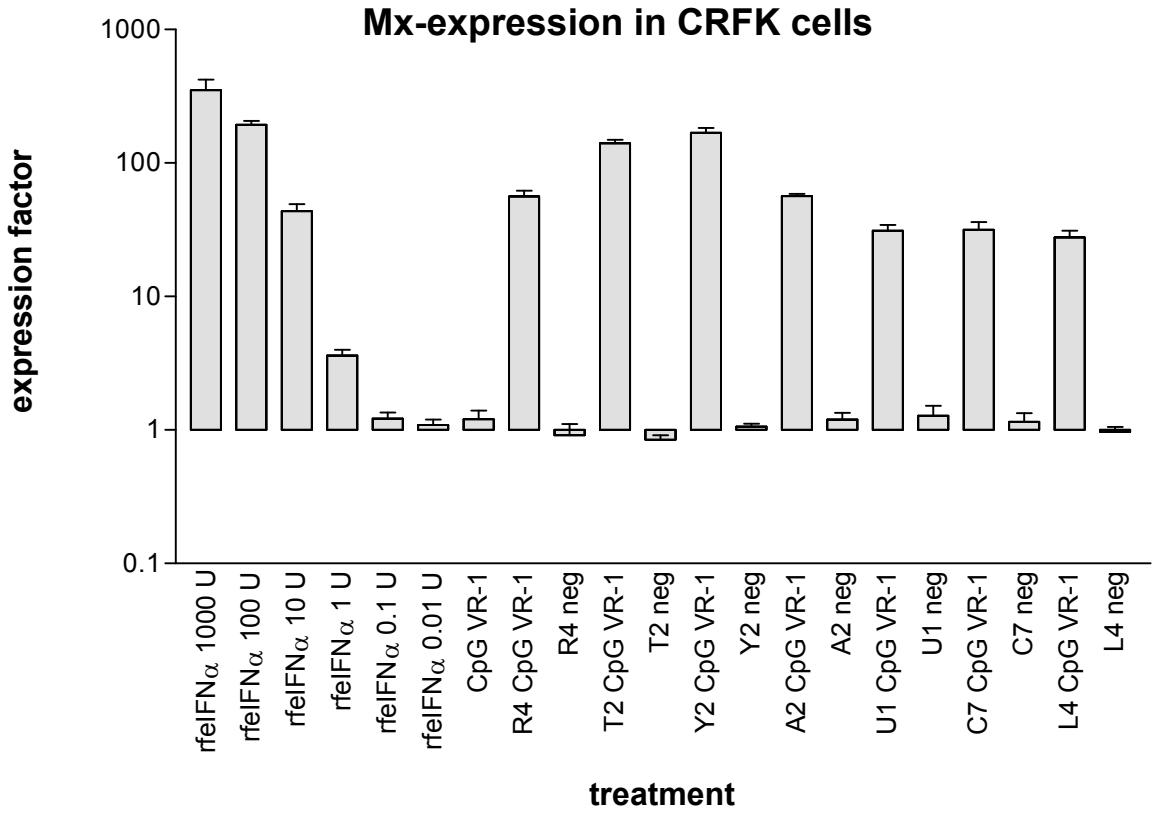


Figure 13: Mx gene expression factors in CRFK cells treated with supernatants or controls

CRFK cells were treated for 24 hours with a 10-fold serial dilution of recombinant feline IFNα, CpG VR-1 or supernatants derived from PBMCs of different cats (R4, T2, Y2, A2, U1, C7, L4) treated with CpG VR-1 or medium alone (neg). mRNA expression of Mx gene was measured by real time PCR and normalized to two feline housekeeping genes (GUSB and YWHAZ). Expression factors were calculated by GeNorm comparing results of treated to untreated samples. The average of duplicate CRFK cell cultures is presented for each treatment.

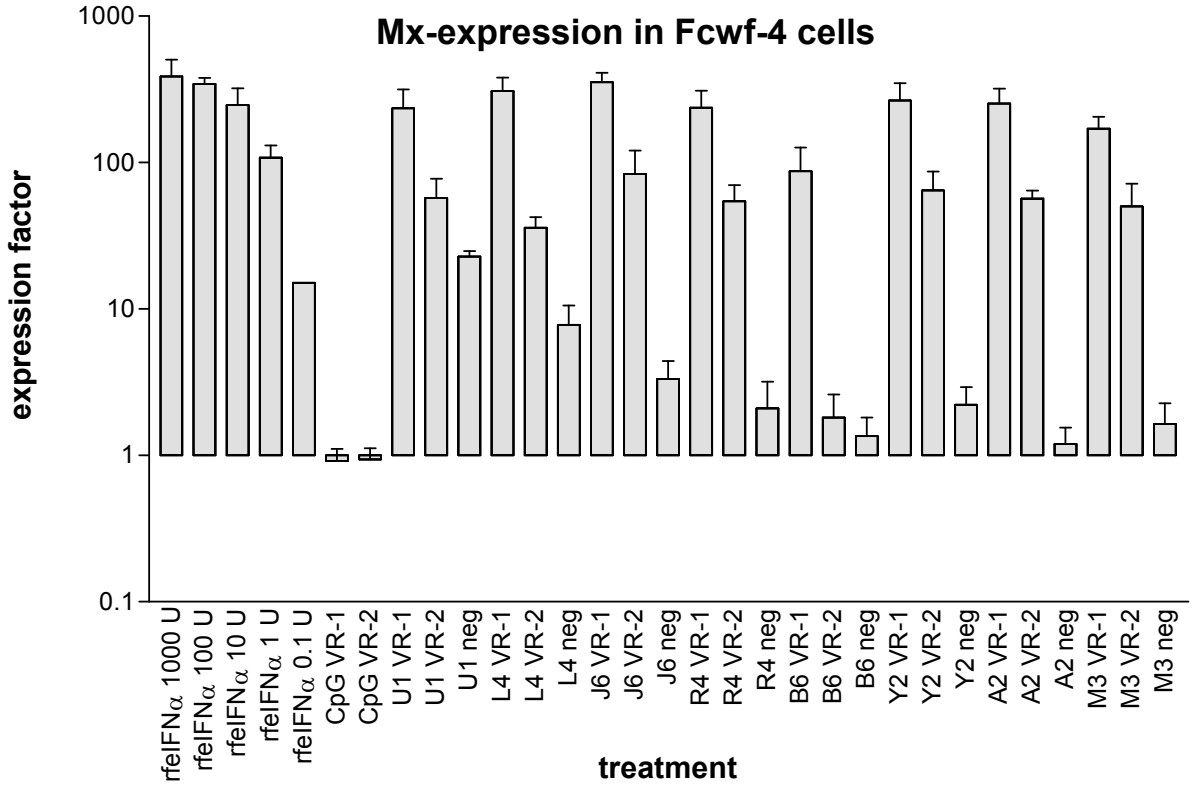


Figure 14: Mx gene expression factors in Fcwf-4 cells treated with supernatants or controls

Fcwf-4 cells were treated for 24 hours with a 10-fold serial dilution of recombinant feline IFNα, CpG VR-1, CpG VR-2 or supernatants derived from PBMCs of different cats (U1, L4, J6, R4, B6, Y2, A2, M3) treated with CpG VR-1, CpG VR-2 or medium alone (neg). mRNA expression of Mx gene was measured by real time PCR and normalized to two feline housekeeping genes (GUSB and YWHAZ). Expression factors were calculated by GeNorm comparing results of treated to untreated samples. The average of duplicate Fcwf-4 cell cultures is presented for each treatment.

The expression factor of the Mx gene in CrFK cells stimulated with supernatant from CpG VR-1-treated and non-treated PBMCs is directly correlated to the expression factors of IFN type I in the corresponding PBMCs but not with IFN type II (Figure 15). In the PBMCs treated with CpG VR-1, CpG VR-2 and medium (=neg), the expression factors of only IFNα were measured. IFNα expression could be directly correlated to the induction of Mx, achieved by the corresponding supernatants in Fcwf-4 cells (Figure 16).

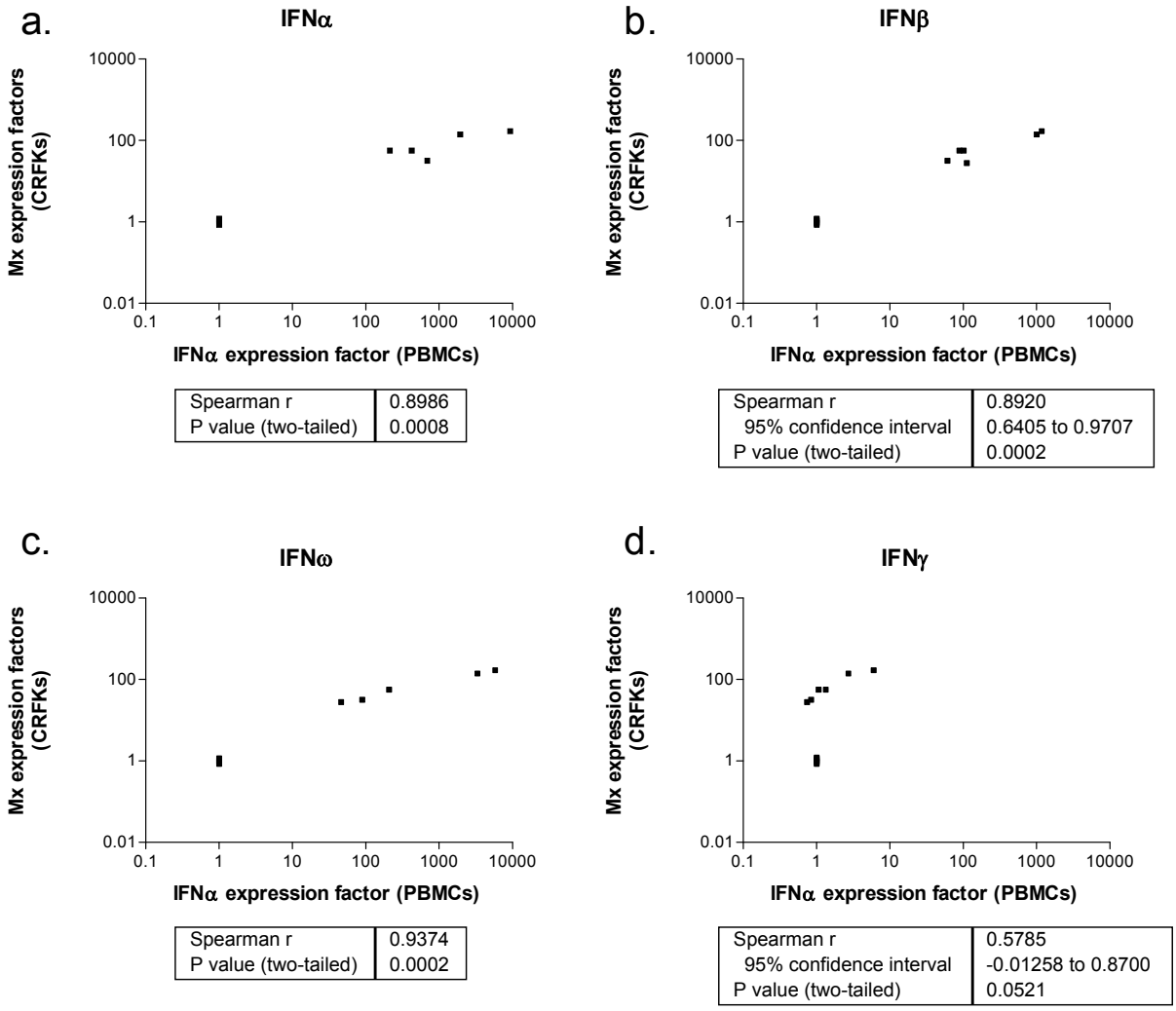
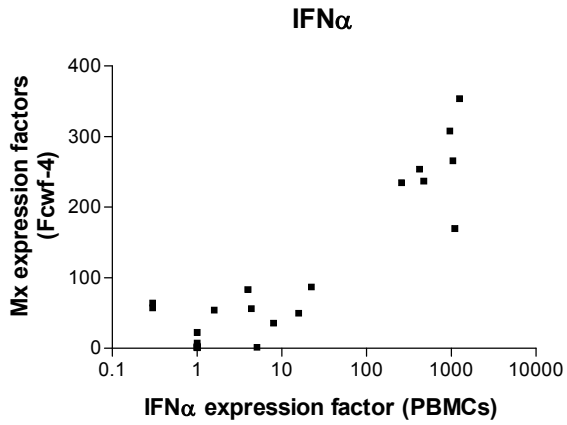


Figure 15a-d: Correlation between expression factors of type I IFN in PBMCs and Mx in CRFK

Mx gene expression factors in CRFK cells after stimulation with supernatants derived from PBMCs treated with CpG VR-1 or medium alone were tested with GraphPad Prism Version 3.0 for correlation to expression factors of type I interferon (a: IFNα; b:IFNβ; c:IFNω) and type II interferon (d: IFNγ) genes in the corresponding PBMCs. Each dot represents one cat.



Spearman r	0.7284
95% confidence interval	0.4501 to 0.8777
P value (two-tailed)	P<0.0001

Figure 16: Correlation between expression factors of IFNα in PBMCs and Mx in Fcwf-4

Mx gene expression factors in Fcwf-4 cells after stimulation with supernatants derived from PBMCs treated with CpG VR-1, CpG VR-2 or medium alone were tested with GraphPad Prism Version 3.0 for correlation to expression factors of the IFNα gene in the corresponding PBMCs. Each dot represents one cat.

5.6. Virus titration

CrFK cells grown to 80-90% confluency in 96-well plates were inoculated in quadruplicates with five-fold dilutions of FCV or FHV stock virus starting with undiluted virus. CPE was measured with the Plaque Assay (Vogel et al. 2001) and calculated using Equation 1. Results are indicated in Table 10 and Table 11. For further experiments on CRFK cells FCV and FHV stocks were both diluted 1:25. The viruses were also titrated on Fcwf-4 cells and for later experiments, FCV stock was diluted 1:5 (Table 12) and FHV 1:25 (Table 13). VSV was previously titrated with the same method and used for experiments in this study in a dilution of the virus stock of $1:10^6$ on CrFK cells and $1:10^5$ on Fcwf-4 cells, respectively.

Table 10: Plaque assay results of FCV titration on CRFK cells

FCV dilution	Mean (OD values)	Std dev.	CPE (%) of VC
5^{0}	0.056	0.020	100
5^{-1}	0.067	0.026	98
5^{-2}	0.073	0.022	96
5^{-3}	0.164	0.070	78
5^{-4}	0.133	0.027	84
5^{-5}	0.193	0.065	72
5^{-6}	0.200	0.060	70
5^{-7}	0.335	0.109	42
5^{-8}	0.398	0.179	29
neg.C	0.540	0.142	0

Table 11: Plaque assay results of FHV titration on CRFK cells

FHV dilution	Mean (OD values)	Std dev.	CPE (%) of VC
5^{0}	0.050	0.010	100
5^{-1}	0.044	0.007	104
5^{-2}	0.053	0.012	98
5^{-3}	0.092	0.013	74
5^{-4}	0.088	0.005	76
5^{-5}	0.119	0.016	57
5^{-6}	0.130	0.022	50
5^{-7}	0.151	0.020	37
neg.C	0.210	0.049	0

Table 12: Plaque assay results of FCV titration on Fcwf-4 cells

FCV dilution	Mean (OD values)	Std dev.	CPE (%) of VC
5^{0}	0.006	0.014	100
5^{-1}	0.003	0.003	101
5^{-2}	0.118	0.078	76
5^{-3}	0.187	0.123	61
5^{-4}	0.364	0.051	24
5^{-5}	0.413	0.047	13
5^{-6}	0.440	0.051	7
5^{-7}	0.408	0.042	14
neg.C	0.474	0.029	0

Table 13: Plaque assay results of FHV titration on Fcwf-4 cells

FHV dilution	Mean (OD values)	Std dev.	CPE (%) of VC
5^{-1}	0.070	0.020	100
5^{-2}	0.090	0.046	96
5^{-3}	0.141	0.079	86
5^{-4}	0.360	0.064	44
5^{-5}	0.459	0.090	25
5^{-6}	0.502	0.076	16
5^{-7}	0.523	0.087	12
5^{-8}	0.632	0.095	-9
neg.C	0.586	0.069	0

Table 10-13: FCV and FHV were titrated by inoculation of either CRFK or Fcwf-4 cells with 5-fold serial dilutions of virus stock for 24 hours. The titrations were performed in quadruplicate for each dilution. The Plaque Assay (Vogel et al. 2001) was performed and the density (OD values) of surviving cells was measured spectrophotometrically. CPE was defined on a scale ranging from 0% (neg. control) to 100% (effect of the lowest virus dilution) for each virus/cell type combination.

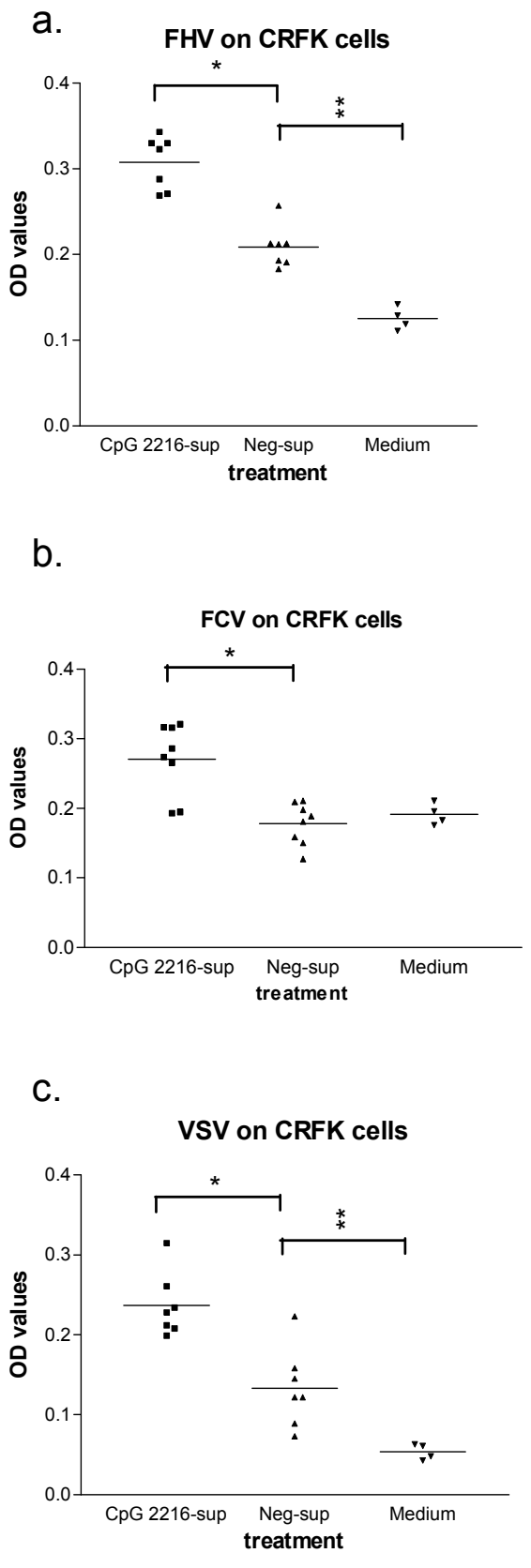
5.7. In vitro viral inhibition assays

CRFK cells or Fcwf-4 cells pretreated with supernatants derived from CpG-stimulated or unstimulated PBMCs of 7-8 cats were infected with, either, FHV, FCV or VSV. All PBMC supernatants were tested for antiviral properties in duplicates. The plaque assay (Vogel et al. 2001) was performed and the absorbance representing surviving cells was measured spectrophotometrically on a SpectraMax Plus384 (Molecular Devices, Bucher Biotec AG, Basel, Switzerland) microtiter plate reader.

In CrFK cells, all tested viruses were significantly inhibited by CpG-supernatants compared to negative supernatants. Inhibition of FHV and VSV was also significantly higher in cells treated with negative supernatants than in the medium-treated positive controls (Figure 17). In Fcwf-4 cells, there were

significant differences in the FCV and FHV replication measured after treatment with CpG VR-1-supernatants when compared to treatment with CpG VR-2- or negative supernatants. CpG VR-1-supernatants also inhibited VSV replication significantly when compared to negative supernatans but not when compared to CpG VR-2-supernatants. For all viruses there was no difference in replication after treatment of Fcwf-4 cells with CpG VR-2- or negative supernatants (Figure 18).

In the plaque assay of the VSV inhibition experiment where CrFK cells were treated with the various supernatants prior to infection, the obtained OD values showed a significant correlation to the Mx gene expression factors induced by the same supernatants in these cells (Figure 19). In contrast, a significant correlation was observed in Fcwf-4 cells between Mx gene expression factors after their treatment with supernatants and the OD values measured in the plaque assays of all three viruses (Figure 20).



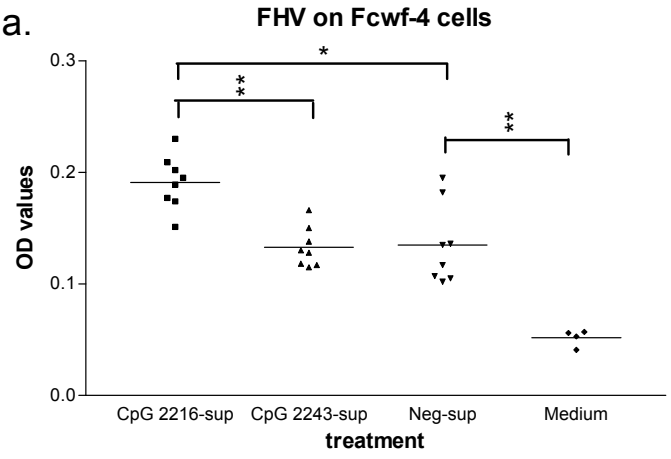
* $p < 0.05$

** $p < 0.01$

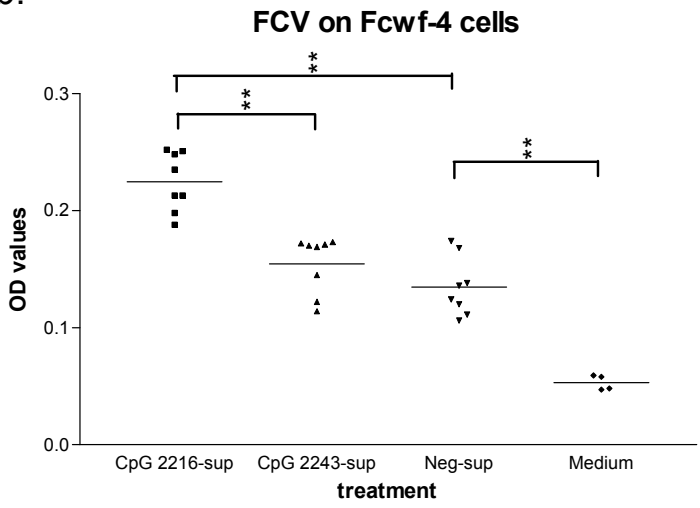
Figure 17a-c: Viral inhibition assays on CRFK cells

CRFK cells had been pretreated for 24 hours with supernatants derived from feline PBMCs treated with CpG VR-1 or with medium (Neg-sup) or as a positive virus control with medium alone and infected with FHV (a), FCV (b) or VSV (c) at dilutions previously determined to be optimal (Table 10 and Table 11). After 16 hours of incubation, the Plaque Assay (Vogel et al. 2001) was performed and the absorbance (OD values) of surviving cells was measured spectrophotometrically. Each dot represents the mean OD values of two duplicate CRFK cell culture samples treated with the supernatant from PBMCs of one cat. The four CRFK cell cultures treated only with medium, thus representing positive virus controls, are displayed individually. Differences between supernatant treatments (CpG VR-1, Neg-sup) were evaluated with a non-parametric Wilcoxon signed rank test for paired samples and differences between negative supernatants and medium were assessed with a Mann-Withney U-test for unpaired samples.

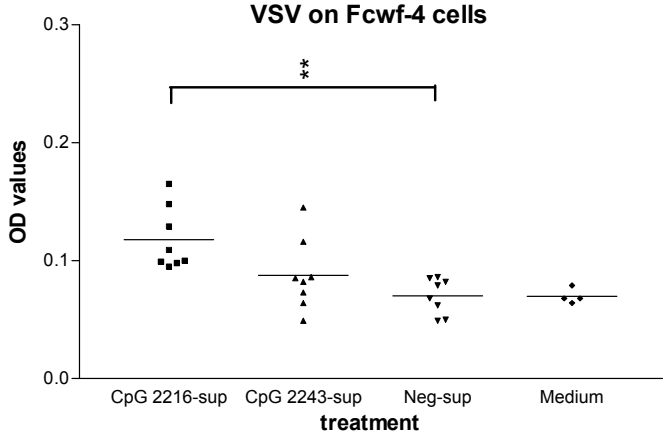
a.



b.



c.

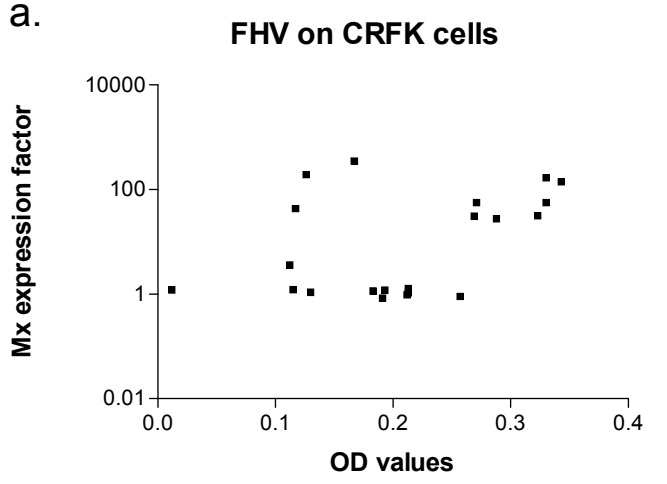


* p < 0.05
** p < 0.01

Figure 18a-c: Viral inhibition assays on Fcwf-4 cells

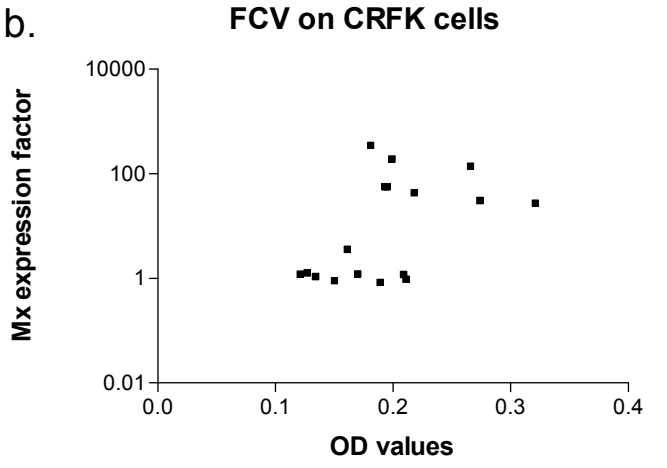
Fcwf-4 cells had been pretreated for 24 hours with supernatants derived from feline PBMCs treated with CpG VR-1, CpG VR-2 or with medium (Neg-sup) or as a positive virus control with medium alone and infected with FHV (a), FCV (b) or VSV (c) at dilutions previously determined (Table 12 and Table 13). After 16 hours of incubation, the Plaque Assay (Vogel et al. 2001) was performed and the absorbance (OD values) of surviving cells was measured spectrophotometrically. Each dot represents the mean OD values of two duplicate Fcwf-4 cell culture samples treated with the supernatant from PBMCs of one cat. The four Fcwf-4 cell cultures treated only with medium, thus representing positive virus controls, are displayed individually. Differences between supernatant treatments (CpG VR-1, CpG VR-2, Neg-sup) were evaluated with a non-parametric Wilcoxon signed rank test for paired samples and differences between negative supernatants and medium were assessed with a Mann-Withney U-test for unpaired samples.

a.



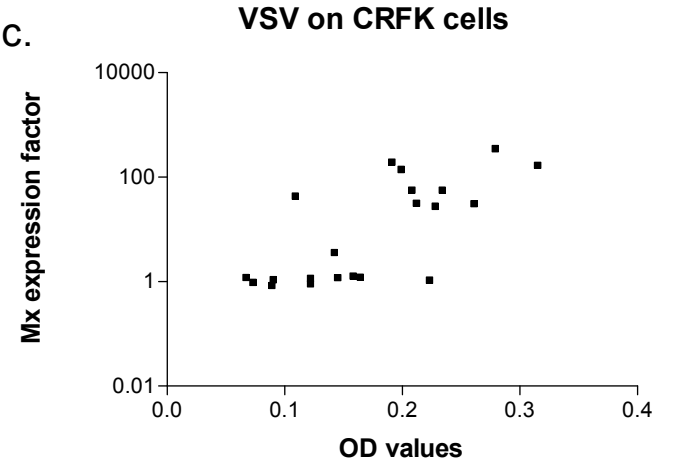
Spearman r	0.2593
95% confidence interval	-0.2074 to 0.6298
P value (two-tailed)	0.2564

b.



Spearman r	0.3676
95% confidence interval	-0.1525 to 0.7283
P value (two-tailed)	0.1466

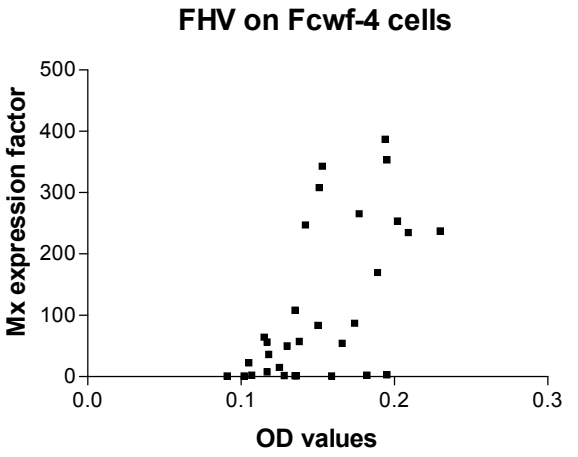
c.



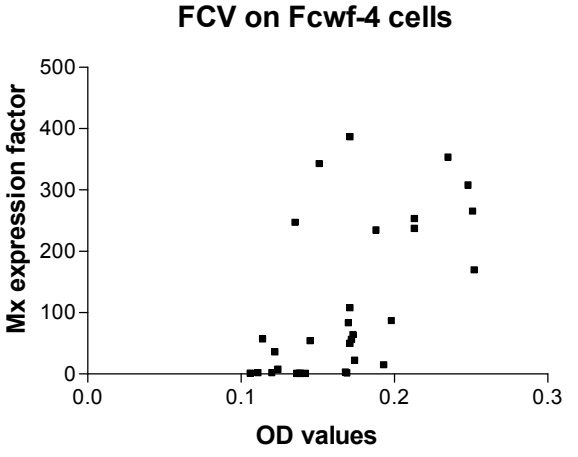
Spearman r	0.6768
95% confidence interval	0.3342 to 0.8615
P value (two-tailed)	0.0008

Figure 19a-c: Correlation of viral inhibition and Mx expression factors in CRFK cells

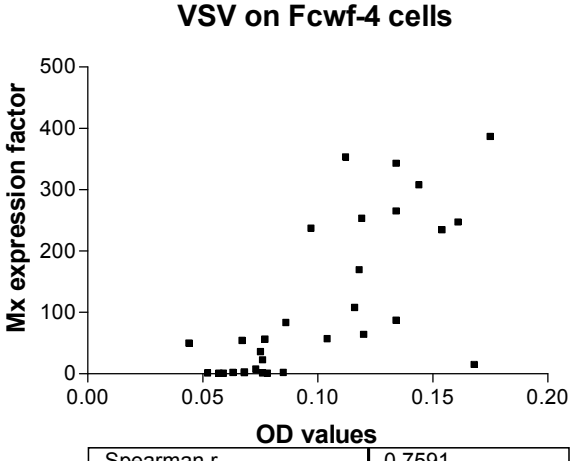
Mx expression factors measured with real time PCR in CRFK cells after stimulation with supernatants derived from PBMCs stimulated with CpG VR-1 or medium were tested for correlation with the OD values measured with the Plaque Assay (Vogel et al. 2001) in CRFK cell cultures pretreated with the same supernatants and infected with FHV (a), FCV (b), VSV (c). Spearman coefficients, confidence intervals and P values were calculated by GraphPad Prism Version 3.0.



Spearman r	0.5689
95% confidence interval	0.2585 to 0.7729
P value (two-tailed)	0.0008



Spearman r	0.6237
95% confidence interval	0.3360 to 0.8049
P value (two-tailed)	0.0002



Spearman r	0.7591
95% confidence interval	0.5460 to 0.8800
P value (two-tailed)	P<0.0001

Figure 20: Correlation of viral inhibition and Mx expression factors in Fcwf-4 cells

Mx expression factors measured with real time PCR in Fcwf-4 cells after stimulation with supernatants derived from PBMCs stimulated with CpG VR-1, CpG VR-2 or medium were tested for correlation with the OD values measured with the Plaque Assay (Vogel et al. 2001) in Fcwf-4 cell cultures pretreated with the same supernatants and infected with FHV (a), FCV (b), VSV (c). Spearman coefficients, confidence intervals and P values were calculated by GraphPad Prism Version 3.0.

6. Discussion

6.1. Background of the study

6.1.1. Objectives

The objectives of this study were 1) to establish diagnostic tools to measure the relative expression of immunologically important genes during the innate and early adaptive immune response in the cat, 2) to characterize the innate immune response upon stimulation of feline PBMCs with an ODN containing CpG type A motifs, and 3) to determine whether resistance to FCV and FHV in feline cell lines can be induced using supernatants of PBMC cultures treated with the CpG-containing immune response modifier CpG VR-1.

6.1.2. Context of the study

New emerging or rapidly changing viruses represent a great challenge in human and veterinary medicine, as the development of new and safe vaccines is extremely time-consuming, allowing meanwhile the dissemination of the pathogen. In this context, the stimulation of the innate immune system represents an alternative method to specific immunization. The possibility to induce an enhanced resistance against viral threats by stimulating the innate immunity would be of some importance, especially in the face of an impending pandemic. For cats, the availability of a pre-treatment before they are temporarily brought into shelters or catteries would be an excellent benefit. Due to the narrow contact of cats from different backgrounds in these establishments, the infectious pressure is much higher than in the normal environment of a pet cat. Additionally, many feline viruses can induce latent, asymptomatic carrier states and can be reactivated in highly stressful situations, favouring the transmission of various feline diseases. The risk is particularly high with FCV, which is extremely persistent in the environment and can contaminate, for example, cages and feeding devices, leading to possible indirect transmission of this virus.

The stimulation of the innate immune system, the first line of defence against microbial agents, could induce an immediate broad-spectrum response, preparing and strengthening the host's ability to fight and overcome viral infections at an early time point. Early response against invading pathogens relies on the recognition of distinct microbial components, called pathogen-associated molecular patterns (PAMPs), by pathogen recognition receptors (PRRs), such as the Toll-like receptors (TLRs) and subsequent downstream signalling which results in an enhanced expression of type I IFNs and other cytokines, thus inducing an antiviral state.

With recombinant type I IFNs, prophylactic and therapeutic successes have already been achieved against several viral diseases in humans (for example used in the standard therapy against hepatitis C) (Moriyama et al. 2006), mice (e.g. CMV) (Bosio et al. 1999) and other species including cats (FeLV/FIV) (de Mari et al. 2004). In feline cell lines, type I IFN has proven to have antiviral properties against several viruses, such as FHV, rotavirus, FCV, FCoV, and feline panleukopenia virus (Mochizuki et al. 1994). The treatment with a synthetic analog of viral PAMPs, an ODN containing

CpG type A motifs that stimulate TLR9, mimics viral infection and can induce an antiviral immune response by stimulating the expression of type I IFNs and other cytokines.

6.1.3. Hypothesis

In this study we investigated the hypothesis that mRNA gene expression of type I IFNs (IFNα, -β, -ω), Mx, proinflammatory cytokines (IL-6, TNFα) and of cytokines representing an enhanced Th1 polarization and NK cell activation (IL-12, IL-15, IFNγ) would occur preferentially after stimulation of PBMCs with CpG VR-1. Furthermore, the antiviral factors induced by the stimulation of feline immune cells were anticipated to promote resistance to FHV and FCV infections in vitro.

6.2. Design of the study

In order to investigate the systemic response upon immune stimulation with a CpG containing ODN immune response modifier in vitro, it appears reasonable to work with a mixed population of immune cells, and to create an environment favourable to multiple cellular and molecular interactions. Peripheral blood mononuclear cells (PBMC) fulfil at best these requirements. This cell population not only comprises plasmacytoid dendritic cells (pDC), which have been shown in mouse and human models to be the major producers of IFN type I, but also other immune cells such as, cDC, NK cells, monocytes, B and T lymphocytes, which are activated by INF-α and themselves produce further cytokines in addition to carrying out their other immunologic functions.

A type A CpG-ODN, which has been shown to induce high levels of type I IFN in mouse and human cells (Krug et al. 2001), was chosen for this study over a type B CpG-ODN that would more specifically induce IL-6 and IL-12.

For the characterization of the innate immune response upon CpG stimulation the following cytokine real-time PCR assays were chosen for mRNA expression measurements: IFNα, -β, and -ω were used to measure the type I IFN response. Analysis of Mx gave indications on the presence of biologically active type I IFNs and their subsequent signalling in target cells. IL-6 and TNFα were used to display proinflammatory reactions. IL-12 and IFNγ were chosen to characterize Th1 polarization while IL-4, on the other hand, illustrated a Th2 polarization. IL-10 was chosen as a marker for immune regulatory mechanisms. Finally, IL-15 was used as a marker for NK cell stimulation whereas IFNγ, Perforin and Granzyme B were thought to give indications on effector and cytotoxic capacities of these cells. Finally, in order to more precisely characterise the cellular response during CpG-ODN stimulation, TLR9 expression was additionally measured.

6.3. Results

6.3.1. TaqMan® real-time PCR assays

All optimized TaqMan® real-time PCR assays resulted in adequate amplification efficiencies ranging from 0.91 to 1.15 with good correlations (correlation coefficients between 0.89 and 0.998). With many

systems, a slight inhibition of the amplification could be observed in undiluted samples and to a lesser degree in the lowest dilution (1:5) resulting in calculated amplification efficiencies above 1. For this reason most cDNA samples were diluted before PCR measurement in the various experiments of this study.

6.3.2. Selection of two housekeeping genes

For the determination of relative gene expression factors, accurate normalization of real-time PCR results with co-analyzed internal reference genes, referred to as housekeeping genes, is of profound importance. These housekeeping genes should be expressed stably at an adequate level and independently of the experimental setting and should not be co-regulated with the target gene to be measured. Expression levels and stabilities of potential candidate genes have been shown to vary greatly among different tissues (Kessler et al. 2009). Therefore, in this study, the stability of several housekeeping genes was evaluated in CRFK cells and PBMCs with two calculation programs (GeNorm and BestKeeper). The ranking of the expression stability yielded by these two methods differed considerably.

In both experiments conducted for the selection of the best suited housekeeping genes (described in section 4.8) GeNorm calculations indicated that two reference genes were sufficient for accurate normalization (V2/3 < 0.15; data not shown).

Of the five tested housekeeping genes GUSB was found to be the most stable in two and the second stable in one out of four calculations. YWHAZ ranked second in three of four calculations and RPS7 was three times third and once fourth. ABL was excluded as a possible reference gene for this experiment as it ranked last in three out of the four calculations. The expression levels of ACTB were much higher than those of the other housekeeping genes. For optimal normalization, Kessler et al. (2009) recommended, the use of housekeeping genes with similar expression levels to that of the target genes. The expression levels of cytokines, especially in unstimulated cells, tend to be low, rendering ACTB undesirable for normalization in these experiments. Thus, GUSB and YWHAZ were chosen as reference genes for normalization in further experiments in this study.

6.3.3. Cytokine gene expression in stimulated PBMCs

Due to the lack of available antibodies against feline IFNα and other feline cytokines, all cytokine measurements and calculations of expression factors were conducted at the mRNA level. Since it is unknown to us when exactly the highest amount of protein is liberated into the supernatant, the maximal induction of mRNA was taken as an indication for optimal stimulation in all experiments in this study.

6.3.3.1. Evaluation of optimal CpG concentration

In order to determine the amount of CpG VR-1 for optimal stimulation of PBMCs, four different concentrations were evaluated. The tested concentrations included an approximation of the final concentration recommended in a previous publication (Hansmann et al. 2008) and 2x, 4x and 8x of that concentration. The gene expression factors of IFNα and IFNω were measured to estimate the

induction of type I IFN, and those of TNFα enabled to asses the proinflammatory response. The concentration 2x induced higher amounts of both IFNα, and IFNω than 1x, whereas 4x only induced slightly more IFNα than 2x but less IFNω. The expression of TNFα on the other hand, did not change noticeably with raised CpG concentrations. Therefore the final concentration 2x (= 5 µg/ml) of CpG VR-1 was optimal for the stimulation of the innate immunity in feline PBMCs.

6.3.3.2. Evaluation of optimal CpG incubation time

In order to find the optimal duration of CpG treatment for maximal innate immune stimulation of feline PBMCs, incubation times of 24, 32, 56, 104 and 128 hours were evaluated. Earlier time points were not considered due to impracticability reasons for further experiments. Besides IFNα, IFNω and TNFα, the expression factors of IL-6, IFNγ and IL-12 were additionally measured.

The highest induction of IFN type I expression could be detected already after 24 hours. A decrease of both IFNα and IFNω could be seen at the time point of 32 hours and after 56 hours the expression levels returned to those before treatment. TNFα, IFNγ and IL-12 were also maximally expressed after 24 hours. IL-6 on the other hand showed a small peak after 56 hours. Thus, 24 hours of incubation were found to be optimal and used in all later experiments.

6.3.3.3. Main experiment: Expression of 14 genes in PBMCs of 14 cats

All measured type I IFNs were highly up-regulated after CpG treatment. The type I IFN-inducible Mx gene was also greatly induced, indicating that not only the mRNA expression of type I IFNs was stimulated, but also the production of biologically active type I IFNs able to signal on further target cells.

Two out of the four tested very young cats of age group 1 (3 months old) responded quite conversely concerning their type I IFN response to CpG stimulation. In these two cats, type I IFN was not up-regulated but rather slightly down-regulated. Consequently, the same applied for the Mx gene. However, the other two cats of that age group showed a type I IFN expression pattern similar to that of the adult cats. Thus, with the exception of IFNβ, no significant differences between the age groups could be detected.

The expression of TLR9 did not differ significantly between stimulated and unstimulated PBMCs. However, again the two young cats mentioned above reacted differently than all the others. In these two cats, a slight up-regulation of TLR9 could be measured, whereas in all other cats a tendency to down-regulation of the expression of this gene was observed. The differences relative to TLR9 between group 1 and the other groups were however not significant. These results raise the question, whether the innate immune response in the two cats was not yet mature and the type I IFN response therefore incomplete, and whether they reacted with an up-regulation of TLR9 because its storage pool in the endosomal reticulum might not have been fully developed yet.

In addition to the up-regulation of type I IFNs, also the proinflammatory cytokines IL-6 and TNFα were significantly induced, although to a far lower degree. The expression of the Th1 stimulating IL-12 was enhanced, whereas Th2 stimulating IL-4 did not show significant changes in its expression pattern. This indicates a T helper cell polarization towards a Th1 immune response, favouring resistance

against viral infections. IL-15, as a marker for NK cell stimulation, was also up-regulated as well as Granzyme B which is produced by these cells in order to perform their cytotoxic function. Perforin, on the other hand, which is also involved in cytotoxic killing, was not induced. IFNγ, which is produced by Th1 cells as well as activated NK cells, was not induced after 24 hours of CpG treatment. It is possible that this period of immune stimulation is too short and the IFNγ producing cells first need to proliferate before significant induction of IFNγ can be detected. Another explanation for this observation could be that these cells additionally require the presence of an antigen for complete stimulation. For further investigations on the stimulation of NK cells, a cytotoxicity assay could be performed (Mickel et al. 1988).

Overall, the CpG VR-1 stimulation of PBMCs provided a cytokine profile favourable for an enhanced resistance and the potential to fight against viral infections.

6.3.4. IFN and Mx mRNA expression in PBMCs

In PBMCs, the elevated expression of the Mx gene was correlated to the induction of gene expression of each measured type I IFN after CpG stimulation but not to that of the type II IFNγ. These observations confirm the findings of various other studies (Staeheli et al. 1986; Der et al. 1998) which explicitly link type I IFN to the induction of Mx production in neighbouring cells. So far no other molecules are known to co-regulate Mx expression.

6.3.5. Mx gene expression in supernatant-treated CRFK and Fcwf-4 cells

When the supernatants of CpG-treated PBMCs were incubated with target cells, namely CRFK cells and Fcwf-4 cells, high expression of the Mx gene was induced. The treatment of these cells directly with the CpG molecule, however, did not have any effect on Mx gene expression. In contrast, when these cell cultures were incubated with a serial dilution of recombinant feline IFNα, there seemed to be a linear relationship between the amount of recombinant IFN used and the expression of Mx, with higher concentrations of rFeIFN leading, as expected, to higher Mx expression. The Mx gene expression was used as a marker to indicate the degree by which type I IFNs, as a consequence of their down-stream signalling, stimulate the production of intracellular antiviral proteins in target cells.

In the experiment with Fcwf-4 cells, supernatants obtained by stimulation of PBMCs with CpG VR-2, the CpG VR-1-control, were also capable of slightly inducing Mx gene expression. Although the differences between the treatment with CpG VR-2-supernatants and negative supernatants were not significant in contrast to the differences between treatments with CpG VR-1-supernatants and CpG VR-2-supernatants, it must be noted that the GC control of CpG VR-1 was not able to completely abolish the immunostimulatory effect of the ODN. A possible explanation could be that this ssDNA molecule behaves differently in individual cats and differently cats from what has been reported for mice. Although CpG motifs seem to be the most important structures, palindromic sequences also play a role in the stimulation of TLR9 and ODNs induce different effects depending on their structure (Dalpke et al. 2004). Additionally, other cell types could be involved and the efficiency of the

stimulation could vary. Further on, other pathogen recognition receptors of the innate immunity might exist inducing IFN upon binding of ssDNA.

The Mx expression in CRFK cells after stimulation with CpG VR-1-supernatant was correlated with the expression of type I IFN genes in the stimulated PBMCs from which the supernatants were derived. There was again no correlation with the expression of type II IFNγ. IFNα expression was also correlated to the Mx expression in Fcwf-4 cells. In the corresponding PBMCs of that experiment, the other IFNs had not been measured hence no statement can be made about their correlations to Mx, even though it can be expected that they behave in a similar manner than in PBMCs and in CRFKs.

The correlations between type I IFN gene expression measured in PBMCs used for supernatant production and the Mx gene expressions measured in supernatant-treated CRFK and Fcwf-4 cells support the hypothesis of the presence of type I IFN in the supernatants on a protein level. The observed effects after incubation with the supernatants can be compared to those observed after incubation with recombinant feline IFNα. The presence of unknown factors that directly or indirectly affect Mx expression cannot be excluded, but current knowledge supports that the type I IFN in the supernatant should be responsible for the observed effects.

6.3.6. In vitro viral inhibition assays

In 1994, Mochizuki et al. showed antiviral activity of rFeIFN against several feline viruses, including FHV and FCV, in Fcwf-4 and CRFK cells (Mochizuki et al. 1994). Since both of these two cell lines were proven to be sensitive to recombinant IFN, it seemed reasonable to test the antiviral activity of PBMC-supernatants also on these cells. To transfer the supernatants containing all possible effector molecules – whether inhibitory or stimulatory - on these target cell cultures mimics more closely the effects that could take place after systemic administration of the molecule in vivo.

FHV and FCV both belong to the cat flu syndrome causing recurrent upper respiratory track diseases and other disorders thus representing a frequent problem in feline medicine. The vaccines against these two viruses cannot protect cats from the infection nor from becoming long-term carriers. Therefore, as discussed already in chapter 6.1.2, increasing resistance to FHV and FCV infections in the cat by stimulation of the innate immune system could be utterly beneficial. However, induction of resistance to both of these viruses requires the induction of a broad-spectrum antiviral activity, as they greatly differ in morphology and pathology. Our viral inhibition assays allow to measure the ability of the CpG VR-1 molecule to inhibit FCV and FHV replication in vitro by incubation of CRFK and Fcwf-4 cells with the supernatants of CpG VR-1-stimulated PBMCs prior to their inoculation with the viruses. VSV was used as a reference virus since it is well-known to be extremely sensitive to IFNs.

The plaque assay, used to measure viral inhibition in the experiments of this study, depicts cell survival in the separate wells at a precise point in time. In order to detect inhibition of viral replication successfully, several factors had to be taken into consideration: 1) the time of incubation with the treatments before viral inoculation (24 hours had been evaluated as the time point of maximal mRNA expression of genes of interest of the innate immune system. In view of the good results in experiments in which Mx was measured, the incubation time wasn't further optimized for viral inhibition experiments), 2) the viral dilution utilized for inoculation of the cells (the highest dilution capable to

infect 100% of the wells was evaluated in viral titration experiments for both cell lines), and 3) the time point at which cell culture should be interrupted to perform the plaque assay (see below).

Starting 14 hours post inoculation, the cells from each experiment were examined microscopically and evaluated for CPE at an hourly interval. Uninfected control wells were thus regularly checked to form continuous monolayers. The plaque assay was performed when 80-90% of the cells in the virus control wells indicated CPE, namely at 16 hours post inoculation in experiments with both CRFK and Fcwf-4 cells. Performing the plaque assay at this time point is essential to detect eventual differences in CPE between treated cells and untreated cells. This difference becomes more difficult to measure when positive control wells reach 100% CPE and viral replication stops due to lack of surviving cells, while it continues in treated wells.

Compared to the method described by Vogel et al. (2001), the cell fixation step with formalin was extended to 40 min in order to ensure a firm attachment of the cells to the bottom of the wells, all other steps were performed as described (Vogel et al. 2001).

CpG VR-1-supernatant was able to inhibit not only the IFN-sensitive VSV, but also FHV and FCV in both cell lines significantly compared to the negative supernatant. Even in the CRFK cells, which had previously been shown to be less sensitive to rFeIFN than Fcwf-4 cells (Mochizuki et al. 1994), good results were achieved.

CpG VR-2-supernatant, used as a CpG control in experiments on Fcwf-4 cells, induced effects similar to those of the negative supernatant. The differences in antiviral action of the CpG VR-1-supernatants and the negative supernatants were therefore specific for the CpG motifs. Direct incubation of the CpG molecule with target cells prior to infection, however, did not inhibit virus replication in Fcwf-4 or CRFK cells; it even seemed to tend to the contrary by slightly enhancing CPE (data not shown). This indicates that the inhibiting properties were actually achieved by the factors produced by the PBMCs upon CpG stimulation and not by possible interactions of residual CpG molecules in the supernatant with the virus or the target cells. The difference in viral inhibition between the treatment with negative supernatants and medium could be due to unknown factors that are present in the negative supernatant and were produced by the PBMCs either during the purification procedure or during culture when left unstimulated and cultivated in medium alone. This inhibition was not as pronounced as that observed when target cells were treated with CpG VR-1-supernatants, however in four out of six experiments a statistical difference in viral inhibition was measured between treatment with negative supernatants or medium alone.

The OD values obtained in the plaque assay for FHV, FCV and VSV in Fcwf-4 cells correlated significantly with the expression of Mx factor induced by the same supernatants, showing a direct relationship between type I IFNs present in the supernatants and their antiviral activity. In the CRFK cells this relationship was not as apparent and only significant for VSV. Since the supernatants nevertheless showed antiviral activity, CRFK cells might be more influenced by other, unknown factors in the supernatants.

7. Conclusions

CpG VR-1 was able to highly up-regulate the gene expression of type I IFNs and Mx, to induce the proinflammatory cytokines IL-6 and TNFα and enhance the expression of IL-12 and IL-15 as well as of Granzyme B in feline PBMCs after in vitro stimulation. The PBMCs produced in consequence amongst other factors biologically active type I IFNs which were released into the cell culture supernatant. The simulation of the target cells (CRFK and Fcwf-4 cells) with these supernatants was able to up-regulate Mx gene expression and to inhibit the replication of FCV, FHV and VSV.

Our results indicate significant biological activity of CpG VR-1 in the cat. The possibility to effectively stimulate the innate immune system in this outbred species renders the cat a much more suitable model for human research on the innate immune system than inbred laboratory mice. This supports the further development of the feline model for such studies. The CpG VR-1 molecule could be for example used in vivo as a stand alone agent to stimulate innate immunity in order to enhance resistance against viral infections or as an adjuvant in vaccines since several reports indicate that adding CpG ODN significantly boosts vaccine immunogenicity in different species (reviewed in Klinman et al. 2009). Future in vivo studies should enable to evaluate the medical safety and tolerability as well as the efficacy of cytokine responses before performing experiments with viral challenges.

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